

NSF boost sends the right message to Congress

Clinton's belated decision to invest heavily in basic science and engineering at the National Science Foundation deserves to win the support of his political opponents on Capitol Hill.

The United States, as Winston Churchill once observed, can usually be relied upon to do the right thing — after exhausting all of the other options available. The aphorism applies neatly to the research component of President Bill Clinton's eighth and last federal budget, which was delivered to the Congress on Monday.

The budget proposal would invest an additional \$2.8 billion in research and development, with most of the money being made available for university grants from the National Science Foundation (NSF) and the National Institutes of Health (NIH), the two agencies with the strongest track record of stringent peer review (see page 585). It also incorporates two major initiatives — in nanoscience and information technology — that will again place heavy emphasis on basic research conducted in universities.

The Clinton administration has acknowledged the need to shift the United States' massive research and development portfolio in the direction of peer-reviewed university research almost from the outset. *Science in the National Interest*, a report published by the White House Office of Science and Technology Policy in 1994, endorsed such a shift as the best way to extract maximum value from research dollars.

But the administration's economic advisers always held technology development efforts as the top priority in its research and development portfolio, and much political effort went into trying to expand such programmes, in the face of justified congressional scepticism. Less politically contentious, but equally insidious, was the administration's tendency to maintain support for intramural programmes,

however unproductive, in government laboratories and research centres. Progress in strengthening non-biomedical university research has therefore been unnecessarily slow, and it is only now, with extra money available for government expenditure as a whole, thanks to the budget surplus, that decisive action can be taken to expand the NSF.

Not everyone agrees that this extra money is available, of course. Much of the \$40 billion of extra spending for the fiscal year 2001 contained in Clinton's budget proposal will be fiercely contested in the Congress, where some have already dismissed it as a pre-election spending binge.

But the research component of that plan is in pretty good shape. The NIH will, as usual, do better than the president suggests. And support for the NSF has broadened over the past three or four years. It used to struggle in the Congress because it has no big laboratories, and thus no natural constituency. But fiscal conservatives appreciate the NSF's efficiency and, provided its director, Rita Colwell, can persuade Congress that the nanoscience and information technology initiatives are consistent with NSF standards, its budget prospects are good.

The same cannot be said for the Department of Energy's component of the budget proposal. Most of that is for energy-related research, which the Republicans loathe, and for the Spallation Neutron Source, which they have chosen to associate with the political fortunes of Al Gore, the likely Democratic nominee in November's presidential election. But whoever wins that election will inherit a strong research portfolio that, one way and another, has strong bipartisan support. ■

Think globally, act cautiously

Renewed interest in a scientific approach to global problems is welcome, but needs sensitive treatment.

One of the most significant aspects of this year's World Economic Forum, the annual meeting of top politicians and business leaders that took place in Davos, Switzerland, last week, was the unusually high attention given to scientific and technological issues. Their prominence, expressed in forums and debates on topics ranging from childhood vaccination programmes to the neurosciences, provided a welcome recognition of the extent to which modern science now touches all aspects of our daily lives. There was also recognition — for example, in a surprise decision by business participants to put global warming at the top of their list of pressing global problems — that this involvement can be double-edged.

Behind the scenes, significant progress has been reported on discussions intended to place such debates on a more rigorous scientific footing. In particular, invited representatives of a selection of world scientific academies used the occasion to move forward plans, championed in particular by the US National Academy of Sciences, to create an Inter-Academy Center responsible for giving top-level, impartial scientific advice to governments on issues of global importance. A working group was set up to produce guidelines for creating such a

centre. These will be debated — and hopefully approved — at a full meeting of scientific academies in Tokyo in May.

Any bid to inject greater scientific understanding into issues of global concern is to be welcomed. More would be achieved in mitigating the impact of global warming if there was a broader acceptance among political leaders of the scientific consensus on the potential dangers ahead. And the damaging spread of unscientific medical heresies, such as some alleged hazards of vaccination, continue to plague efforts to promote preventive health strategies in the Third World.

If there is a danger, it is that the agenda implicitly conveyed by this drive for more scientifically based decision-making may appear to be driven primarily by the most powerful actor on the international scene, the United States. Any initiative must reflect a plurality of approaches, not one that reflects the way such issues are seen from a single vantage point. Some are already nervous about the increased US presence in the International Council for Science (see page 582). Such fears may well prove unfounded. But given the power the United States wields as the world's strongest economy, it is essential that it — and its scientists — wield this power sensitively. ■





Lubchenco
First woman to head
International
Council for Science
p582



NASA
Projects delayed for
review after string of
mistakes
p583



SLAC
California's BaBar
looks like winning
the B-meson race
p586



NEAR thing
Valentine's day
asteroid rendezvous
is on the cards
p589

CERN claims first experimental creation of quark-gluon plasma

Munich

The European Laboratory for Particle Physics (CERN) plans to announce today (10 February) that it has "compelling evidence" that its scientists have created the quark-gluon state of matter predicted to have existed shortly after the Big Bang.

If confirmed, this would be the first time that conditions within the first three minutes after the Big Bang — the point at which the protons and neutrons that make up atomic nuclei came into being — have been observed under experimental conditions.

Such 'nucleons' are made up of two types of elementary particle: quarks and the carriers of forces that bind them, gluons. But free quarks have never been detected — theory predicts that they exist only in an unconfined state either during this short window of time, or at energy densities encountered in very energetic heavy-ion collisions.

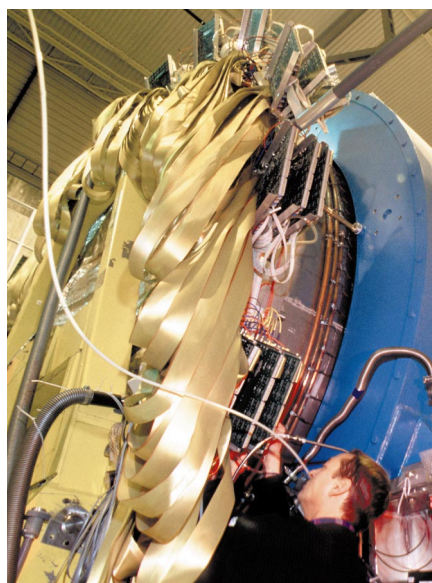
The theory of these particles, quantum chromodynamics, predicts that, at such energies, quarks and gluons coexist unbound within a plasma. Scientists have tried for years to find such a plasma, but the complexity of the phenomena produced by high-energy collisions of massive ions has made it impossible.

CERN scientists now say they have created quark-gluon matter in a series of experiments begun in 1994, in which CERN's Super Proton Synchrotron (SPS) fired very-high-energy beams of lead ions into gold or lead targets.

The collisions create temperatures 100,000 times hotter than the centre of the Sun, and the highest energy densities (3–4 GeV per cubic femtometre) ever reached over a large volume in laboratory experiments. Such collisions should be sufficient to break down the forces that confine quarks inside nucleons.

As the energy levels in the CERN experiment, known as the SPS Heavy Ion Programme, were insufficient to allow free quarks to be detected directly through electromagnetic radiation emitted in the form of photons, CERN scientists also searched for indirect evidence of quark matter.

Detectors in other experiments were optimized for measuring signals from the hadrons, such as protons, neutrons and mesons, that form as the plasma expands and



Detector work: CERN's NA45 experiment has contributed to evidence of quark matter creation.

cools, and the quarks and gluons coalesce.

One experiment was designed to detect the rare J/Ψ meson, made of a charm quark and a charm antiquark. Charm quarks are very heavy, and can be produced only immediately after the lead ion beam hits its target.

Theoretically, the formation of J/Ψ mesons is suppressed by quark-gluon matter, which reduces the interaction between charm quarks and antiquarks. The J/Ψ experiment actually measured a dip in the number of J/Ψ mesons reaching the detector, suggesting that a quark-gluon plasma had been created.

At a conference on quark matter held in Turin last May, the 600 or so physicists from 22 countries involved in the CERN programme decided that their combined results support the theory that quarks are freed up (or 'deconfined') at high energy densities.

They chose to announce their results before the experiments start at the Relativistic Heavy Ion Collider (RHIC), a dedicated quark-gluon plasma factory at the US Brookhaven National Laboratory. When it starts operation in the next couple of months, RHIC's higher energy capacity will allow it to create long-lived quark-gluon

plasma (see *Nature* **400**, 303; 1999).

The announcement appears to be intended to ensure that CERN receives credit for the initial creation of quark matter before the European laboratory's US counterpart announces its own results, which are likely to be more clear-cut.

"Even if the interpretation of some of the individual observations, taken on their own, is controversial, when they are all put together, the evidence that we have been able to create deconfined quark-gluon matter is overwhelming," says Federico Antinori, a particle physicist from Padua, Italy, who is spokesman for one of the CERN experiments.

Reinhard Stock, professor of nuclear physics at the University of Frankfurt, and head of another of the experiments, adds that CERN researchers were able not only to create the quark-gluon matter, but also to observe its decay into protons and neutrons.

CERN's achievement has been welcomed by scientists at RHIC. This facility will begin generating results in early summer, and will study the properties of quark-gluon plasma itself, rather than just its decay. The Brookhaven collider will generate centre-of-mass energies of 200 GeV by colliding two heavy-ion beams, rather than firing one beam onto a fixed target, as occurs at CERN.

Physicists expect that long-lived plasmas will be generated, and that it will be possible to measure the photons generated by free quarks directly. Tom Ludlam, associate director of the RHIC project, says he "agrees completely" that CERN scientists' conclusions that their evidence for a quark-gluon plasma is "very compelling". But he adds that direct measurement of free quarks, as RHIC should allow, would remove any doubts.

CERN will take over from the RHIC experiment when its ALICE experiment (A Large Ion Collider Experiment) begins in 2005. Exploiting CERN's new accelerator, the Large Hadron Collider, ALICE will be able to generate collision energies of 5.5 TeV per nucleon centre-of-mass energy — nearly 30 times that of RHIC.

Alison Abbott

Further reading:
Wilczek, F. Liberating quarks and gluons. *Nature* 391, 330–331 (1998).

Austria taken to court for inadequate laws on animal welfare

Brussels

Austria is to be taken to the European Court of Justice by the European Commission over claims that it has failed to implement adequately European animal-welfare rules on the care of stock animals that are being raised for use in experiments.

The rules form part of directive 86/609/EEC on the "protection of animals used for experimental and other scientific purposes". The commission says that legislation covering the use of animals in experiments in Austria falls well short of the standards required by the directive. The court can order Austria to bring its regulations in line with European Union law, and levy fines of around 100,000 euros (US\$98,000) a day if that ruling is ignored.

The commission says that Vienna has failed to adopt rules in the directive that apply to research centres that breed animals on their own premises, including rules on the general care and accommodation of animals, the role of responsible individuals, and the recording of data on breeding and supplying establishments.

But Andreas Bichl, head of the animal facility at the Research Institute of Molecular Pathology in Vienna, argues that "animals bred as in-house stock are not regulated at present and do not have to be recorded". He argues that such animals are protected under general animal-cruelty laws, but not by specific legislation for experiments.

Austria should have implemented the directive by 1 January 1995, under its terms of accession to the European Union. At the time, the commission concluded that Austria had not complied, and launched an infringement procedure. Austria amended its law, but the commission has now concluded that the reforms were not comprehensive enough.

"If veterinary inspectors want to inspect our facilities, they have to adhere to the laboratory-animals law, which covers only 20 per cent of animals in house," says Bichl. "Mice have a high rate of reproduction and [under the directive] we would have to record every litter; it would be a lot of administrative work."

The commission has also threatened Belgium with court action for allegedly giving "insufficient recognition of data" from experiments in other member states, to avoid unnecessary duplication of animal experiments.

Keith Nuthall

International science council names first female president

Irvine, California

Marine ecologist Jane Lubchenco of Oregon State University will be the next president of the International Council for Science (ICSU), becoming the first woman to head the Paris-based organization.

Lubchenco will take on the duties of president-elect of the organization — formerly known as the International Council of Scientific Unions — in March 2001 under plans agreed last September at the ICSU general assembly in Cairo. She will begin her three-year term as president in 2002 at the next general assembly, scheduled for Rio de Janeiro.

"I am thrilled," says Lubchenco, whose election was announced last week at a meeting of the US National Science Board, on which she also serves. "I consider it a high honour and daunting responsibility. I view science as a key element in enabling a prosperous, secure and healthy future for all of us."

Lubchenco is the Valley professor of marine biology at Oregon State University, where she studies rocky-shore ecology, and will be the first US president of ICSU since 1976. ICSU's board had actively been seeking a US scientist for the nomination this time. "She is the kind of person science needs in a leadership role," says John Boright, executive director for international affairs at the US National Academy of Sciences.

Lubchenco, a former president of the American Association for the Advancement of Science, is experienced in international science diplomacy, and attended last week's meeting of the World Economic Forum in



Lubchenco: expected to keep environmental issues high on the political agenda.

Davos, Switzerland. She hopes to use her ecological background to keep issues such as climate change high on the international political agenda.

ICSU recently named a new executive director, Larry Kohler, an American who has lived in Europe for many years, and who took up his new post on 1 January. ICSU's secretary general is Harold Mooney, a biologist at Stanford University.

There has been unease in some quarters about the ascendancy of Americans in key posts in ICSU. But US officials argue that this could result in greater US support for international scientific efforts.

Rex Dalton

Biotech companies attack NIH rules

Washington

Guidelines on research tools issued recently by the US National Institutes of Health (NIH) came under fire last week from representatives of several small biotechnology companies at a symposium on intellectual property organized by the National Academy of Sciences.

The guidelines advise researchers in general who use NIH money to generate tools — such as reagents and new transgenic technologies — against demanding 'reach-through' rights that claim future intellectual property on products generated from a tool's use (see *Nature* 403, 10; 2000).

But Robert Blackburn, vice-president and chief patent counsel of Chiron, a private biotech company based in Emeryville, California, said that biotech companies need

both reach-through rights and licensing arrangements — without compensation for their intellectual property, they would have a difficult time surviving.

Intellectual property gives biotech companies "the right to be in the market", says Evelyn McConathy, a patent lawyer with the Philadelphia firm Dilworth Paxson LLP, adding that the market, not government policy, should drive licensing deals.

But she admits that a "large outcry" followed DuPont's initial demands that researchers accept reach-through terms if they use Cre-lox, which allows genes to be removed from specific cells and tissues. The outcry forced the company to allow non-profit researchers freely to use the technology, which was developed using NIH funding (see *Nature* 394, 819; 1998).

Paul Smaglik

NASA review leaves projects on launch pad

Washington

All US spacecraft missions scheduled for launch this year are undergoing a sweeping review following a spate of high-profile failures that culminated in the loss of two Mars spacecraft last autumn.

Under the reviews, which have been ordered by Dan Goldin, administrator of the US space agency NASA, teams of NASA-appointed engineers are scrutinizing each project. Two launches have already been postponed, and others may be delayed.

After investigations into the Mars accidents had revealed a series of embarrassing mistakes and management lapses, Goldin directed the heads of his agency's field centres last month to take extra measures to ensure that missions in the pipeline don't have similar problems.

The Jet Propulsion Laboratory in California had already initiated a review of its Mars programme, while the Goddard Space Flight Center in Maryland responded by ordering independent 'Red Team' reviews of each project under its purview.

At least a dozen Earth and space science missions scheduled for launch in 2000 will undergo the review. These include IMAGE, Earth Observing-1, Cluster 2, the High Energy Solar Spectroscopic Imager (HESSI), TIMED, the Microwave Anisotropy Probe and Aqua, the second satellite in the Earth Observing System.

Mission scientists — many of whom are at universities rather than NASA centres — have mixed reactions to the NASA edict. Most say the additional checks can in principle help root out problems. But many worry that adding a major review so close to launch only disrupts projects with very tight schedules and cost margins.

HESSI, for example, is due to launch in July to observe solar flares at high-energy wavelengths. Run by the University of California at Berkeley, the \$72 million Small Explorer mission is a shoestring operation by NASA standards, and has taken just two years to build.

Peter Harvey, the HESSI project manager at Berkeley, estimates that the project has already undergone 20 reviews. "To now have yet another review is killing us," he says, not because of the one or two days taken up by the Goddard team's visit, but because of the two previous weeks his staff will need to prepare for the review.

Just when project engineers should be focused on the launch, he says, they will be presenting graphs instead. "Our attention is turned away from the spacecraft."

A key assumption behind the 'better, faster, cheaper' approach adopted by NASA is that it dispenses with much of the red tape and bureaucratic requirements of past



Delayed exposure: IMAGE is having to wait to study Earth's response to solar magnetic activity.

spacecraft missions, which were a major factor in driving up costs. NASA reviews were to be kept to a minimum. The low-cost missions were also understood to assume a certain risk of failure by not taking the time and money to build in redundant systems.

Some scientists facing Red Team reviews worry that NASA might change its rules in mid-stream following the recent mission failures. The Massachusetts Institute of Technology's \$20 million HETE-2 satellite, for example, was to have been launched in late January from Kwajalein Atoll in the Pacific Ocean. It had already been mated to its Pegasus rocket and was preparing to ship out from California when NASA called off the countdown (see *Nature* 403, 232; 2000).

Congress gets tough with gene therapy

Washington

Legislation to tighten up the supervision of federally funded human gene-therapy trials was introduced into the US House of Representatives last week. The move occurred on the day a Senate subcommittee hearing examined the problems of monitoring such trials.

The hearing was triggered by the case of Jesse Gelsinger, the Arizona man who died four days after receiving experimental gene therapy at the University of Pennsylvania (see *Nature* 401, 517; 1999). But the subcommittee explored other issues concerning clinical research in humans.

According to a spokeswoman, the proposed legislation, introduced by Dennis Kucinich (Democrat, Ohio), was drafted before Gelsinger's death. Under the

Mission rules required that only one tracking station be guaranteed operational for launch. But the space agency decided it wanted two as a precaution. Although there had been no sign of trouble with the spacecraft, it was returned to the east coast for testing, and the launch has slipped to May.

NASA has delayed other launches for relatively minor reasons. IMAGE, which will study the Earth's magnetosphere, is managed by the Southwest Research Institute as the agency's first Midsize Explorer mission. It was to have been launched on 15 February. But after review teams questioned certain technical aspects of the project, the launch slipped to early March.

James Burch, the IMAGE principal investigator at Southwest, expects his spacecraft will ultimately be passed as fit. But he points out that NASA had long ago accepted the inherent risk of fast, cheap missions. "If somebody's going to say we don't want to take the risk of not having significant redundancy in the system, then that puts all these spacecraft in the museum," he says.

Although Burch, Harvey and other project heads say they understand Goldin's concern, they question how much review is enough, and whether the Red Team exercises are productive.

When a spacecraft loses its place in the queue, it can take months to get back to the launch pad. Burch estimates that keeping IMAGE on the ground past its scheduled launch date costs around \$60,000 a day. But, he says, "if NASA is going to pay to slip things to make themselves satisfied that the risk is low enough to go ahead, then that's their prerogative."

Tony Reichhardt

legislation, authority for monitoring federally funded human clinical trials would be transferred from the Office for Protection from Research Risk, under the US Department of Health and Human Services, to an independent agency.

Arthur Caplan, director of the University of Pennsylvania's Center for Bioethics, argued that the rise in clinical-trial holds issued by the US Food and Drug Administration (FDA) indicates a general problem. The FDA issued few clinical holds in the eight years before 1999, but last year stopped at least five trials not involving gene therapy.

At last week's hearing, William Frist (Republican, Tennessee) said that systems to protect human subjects enrolled in clinical trials "are not working", and he was not

► certain that new regulatory bodies would solve the problem.

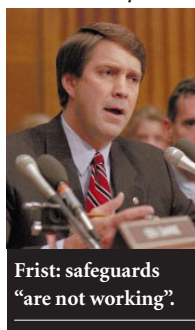
The Gelsinger case revealed a failure to report adverse events in gene-therapy trials. After his death, the National Institutes of Health (NIH) asked researchers using adenoviral vectors — the delivery vehicle used in Gelsinger's trial — to report all adverse events.

Eventually, 652 became public (see *Nature* 403, 237; 2000), including several deaths not directly attributed to gene therapy. Last week's hearing revealed 40 more adverse events, related to vectors other than adenovirus.

Researchers are required to report "serious and unexpected" adverse events only to the FDA, although they must disclose all adverse effects to the NIH's Recombinant DNA Advisory Committee (RAC).

Former RAC chair LeRoy Walters, director of the Kennedy Institute of Ethics at Georgetown University, testified that NIH's 1996 reduction of the RAC's powers had sent a "mixed message" to researchers, possibly indicating that they were no longer required to report to the RAC.

Walters also said that problems in securing a patient's informed consent revealed "a system-wide problem not



Frist: safeguards "are not working".

unique to gene therapy". Paul Gelsinger, the patient's father, testified that researchers had not told his son that two monkeys had died on the experimental treatment.

The researchers had also incorrectly told Gelsinger that a previous subject had benefited from the gene therapy. The two problems were among 18 used by the FDA to justify shutting down five other gene-therapy trials at the University of Pennsylvania (see *Nature* 403, 354; 2000).

More inspections may prevent future problems, said Michael Blaese, chief scientific officer of Kimeragen, a Pennsylvania-based biotechnology company. Blaese, previously a gene-therapy researcher at the NIH, noted that trials on the NIH campus were audited frequently.

Jay Siegel, director of the FDA's Office of Therapeutics Research and Review, testified that the agency typically conducts on-site investigations as a drug or treatment nears approval. He said that the agency has begun to do spot checks at a "very limited" number of clinical sites. "We would like to do more." **Paul Smaglik**

Scientists reject blame for German genome shortfall

Munich

German scientists have rejected claims that lack of government funding for genome research is their own fault, for failing to convince politicians of its importance.

Although funding for the German Human Genome Project (DHGP) is expected to increase substantially next year, it will still fall well short of what many believe the country needs to compete internationally.

Walter Döllinger, a senior official in the research ministry, told a DHGP workshop last month that the scientific community and industry must share the blame for waning political support. Their lobbying activities have been ineffective, he said, and they have not put significant amounts of their own money into genomics research.

Döllinger said the research ministry is trying to maintain political momentum, which has slowed as government priorities changed. The ministry has drawn up a broad strategy paper for genome research that "covers the whole chain of innovation, from basic research to development". He expects it to be approved during the 2001 budget talks in June.

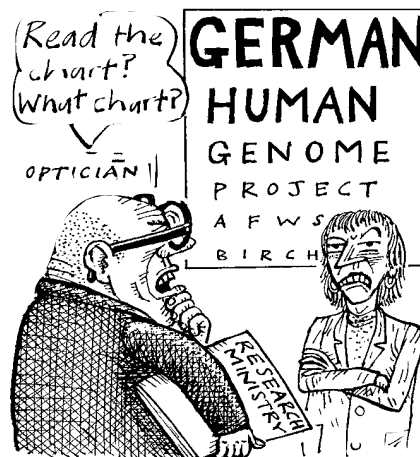
This extends an earlier paper, based on input from the scientific community, that was to have been launched last year. To the dismay of researchers, the paper was shelved, apparently because research minister Edelgard Bulmahn felt that it lacked a sufficiently strong political message to persuade the cabinet to provide the level of extra funding it demanded (see *Nature* 402, 706; 1999).

The present paper brings together all the ministry's activities in genome research, including its new DM100 million (US\$50 million) BioChance programme for start-up companies, along with the activities of research organizations such as the Deutsche Forschungsgemeinschaft (DFG), Germany's main granting agency, and the Helmholtz Society, which runs national research centres.

It proposes a "significant increase" — possibly more than 50 per cent, Döllinger indicated — in project money for the DHGP, currently around DM50 million a year. The paper also proposes setting up a genomics programme for microorganisms and a fund for 'competence centres' to reward interdisciplinary programmes linking research groups from different institutions.

The new package would be more politically convincing than its predecessor, said Döllinger. But researchers say that its scientific aspirations will not be met unless the government supplies a lot more money.

Detlev Ganten, director of the Max Delbrück Centre for Molecular Medicine in Berlin and head of the Helmholtz Society,



rejects Döllinger's claim that scientists have failed to shift funds from old areas of research. The Helmholtz Society has created a small strategy fund in genomics and intends to redistribute its own core funding in favour of genomics, he says. Its genome-related research will be evaluated next month by an international committee. The Helmholtz Society's senate will use the results in its decision on genomics strategies in May.

"The research ministry is very well aware of our activities," says Ganten. "It should not make up excuses to justify its own inactivity. It is naive to say that shifting our money would be sufficient; we need a lot of extra money."

The Helmholtz Society was a co-signatory of a report to the ministry which argued for a tenfold increase in public funding. Other signatories included major research organizations, such as the DFG, and the Förderverein, a consortium of German companies that support technology transfer from the DHGP.

DFG president Ernst-Ludwig Winnacker says he is "surprised" that Döllinger considers scientists' lobbying activities inadequate. He points out that the heads of all German research organizations have written detailed reports and campaigning letters to the ministry, and have launched an initiative to lobby parliamentarians.

"We have taken all opportunities to air the debate in newspapers and the most important political circles," says Winnacker. "Short of hiring a Zeppelin and flying it over Berlin, I'm not sure what more is expected of us."

Werner Schiebler, director of technology licensing and alliances at Aventis, says: "Industry recognizes its responsibility for supporting technology transfer and this is why we support the Förderverein by around DM1.5 million a year." But he adds that industry will only pay for basic research with clearly defined goals. **Alison Abbott**

Clinton's farewell gift to US science agencies

Washington

President Bill Clinton's eighth and last budget proposal — submitted to Congress on Monday (7 February) — would give several US science agencies a larger increase than any of his previous budgets.

"The president has proposed a historic science and technology budget for 2001," said Neal Lane, Clinton's science adviser, setting out details of a package announced by the president in a speech at the California Institute of Technology last month (see *Nature* 403, 349; 2000). "I believe it will generate bipartisan momentum to put the science and technology budget on the right track for future years."

But the budget package as a whole has met with a hostile reception in Congress — where the Republicans control both Houses — as it would raise overall government spending by 4 per cent above previously agreed limits. "The president is proposing the era of big government come back with a vengeance," said Senator Pete Domenici (Republican, New Mexico), chair of the Senate Budget Committee.

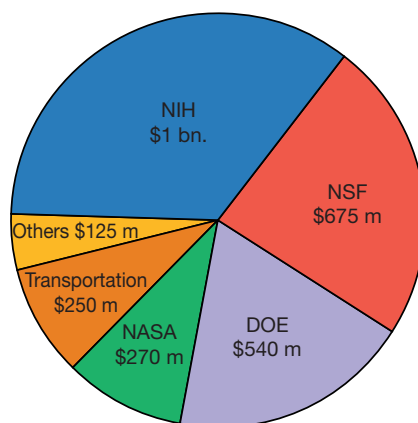
As announced last month, Clinton's budget for 2001 would increase funding for the National Science Foundation (NSF), the biggest funder of non-biomedical research in US universities, by 17 per cent to \$4.6 billion. The National Institutes of Health (NIH) would get a 6 per cent rise, to \$18.8 billion.

It emerged on Monday that the proposal would also raise spending on non-weapons research and development at the Department of Energy (DoE) by 15 per cent, to \$4.2 billion, and at the space agency NASA by 6 per cent, to \$5.2 billion. Of the main science agencies, only the Pentagon has been left out of the bonanza: its proposed budget for basic and applied research would fall by 5 per cent, to \$4.6 billion.

Lane says that the increase at the NSF and other agencies is intended to allow them to catch up with the NIH's rapid expansion. "It begins to restore the balance between biomedical research and the rest of the portfolio," he says.

Half of the NSF's proposed \$675 million increase would be available for general grant awards. "Over \$300 million is not tied to any specific initiative," says NSF director Rita Colwell. "This gives us the flexibility we've been seeking for years."

At the DoE, however, half of the proposed \$340 million increase for non-weapons research is earmarked for the construction of the Spallation Neutron Source at the Oak Ridge National Laboratory in Tennessee. Core programmes in physical research at the department receive small increases of 4 per cent or less.



Jam tomorrow: how Clinton's budget proposal would share money among the science agencies.

Research grants make up \$610 million of the NIH's proposed total increase of \$1 billion. But the president's budget calls for fewer new intramural grants than in each of the past two years. The administration is focusing on continuing funding for grants already made.

Ruth Kirschstein, acting director of the NIH, explains that the NIH is emphasizing new centres through programmes such as the Biomedical Information Science and Technology Initiative, which funds computational biology and bioinformatics. New

money for such centres makes up \$90 million of the agency's increase.

NASA gets a raise for the first time in seven years, with its overall request up 3 per cent to \$14 billion, and projected to climb to \$15.5 billion by 2005. Administrator Dan Goldin will use the money for science and technology, space shuttle safety, and personnel. Space science gets nearly half of this year's \$435 million increase, giving it a budget of \$2.4 billion.

Goldin says he sees NASA "returning to its roots — cutting edge, fundamental R&D". Spending on science, aeronautics and technology, which accounted for only 31 per cent of the agency's budget in 1991, will reach 51 per cent within five years, he says. Goldin and Colwell each hinted that a major collaboration will soon be announced between their respective agencies, probably involving environmental research.

Taking into account the tens of billions of dollars' worth of development work performed by the Pentagon, the final Clinton budget would achieve a target set by the administration in 1993: to spend more on non-military than military research and development. At the height of the Cold War, military spending accounted for 70 per cent of the total, and in 1993 it still outscored civilian research by 60:40.

Colin Macilwain,

Tony Reichhardt and Paul Smaglik

Mostly winners — but the NIF must make do

● **The National Science Foundation** will lead an interagency nanotechnology initiative, which will double research spending in this growing field to \$500 million. Another interagency initiative would boost spending on computing research from \$1.7 billion to \$2.3 billion. The administration says that both projects will support basic research, not technology development.

● **The nuclear weapons research programme** gets a \$300 million boost, taking its budget to \$4.6 billion. But there is no extra money for completion of the troubled **National Ignition Facility (NIF)** at the Lawrence Livermore National Laboratory in California. Bill Richardson, the energy secretary, repeated that any extra funds for NIF must come from Livermore's budget. "The science

is sound" at NIF, Richardson said, "but the management stinks".

● **NASA's hapless Mars programme** gets a much-needed boost: after scraping by on budgets of \$200 million to \$250 million for several years, the multi-mission Mars effort gets \$327 million. Of that, \$35 million will go for 'micromissions' and a Mars telecommunication network described by the agency as "a publicly accessible, live-video 'gateway' to Mars".

● **The biggest space-science initiative** will investigate the Sun and solar storms. NASA's 'Living With a Star' programme — a combination of new missions and enhancements to old ones — will cost \$511 million over five years. Among the spacecraft to be launched are a Solar Dynamics Observatory, Solar Sentinels and a

Geospace Dynamics Network. Most of the money will go to the Goddard Space Flight Center in Maryland.

● **The NIH will get \$47 million** to start building a neuroscience centre on its Bethesda, Maryland, campus (see *Nature* 403, 123; 2000). The project would require another \$26 million in the 2002 budget to complete the first phase of a proposed \$270 million scheme that would include renovations and additions to existing buildings.

● **The Centers for Disease Control and Prevention** would receive a \$202 million increase, including \$70 million for the construction of three laboratories dedicated to research into agents that could be used in bioterrorism and lethal pathogens such as the Ebola virus and hantavirus.

Stanford accelerator takes lead in race to quantify CP violation

Two international teams are competing to measure B-meson decay. Their results will change our understanding of physics and of the Universe.

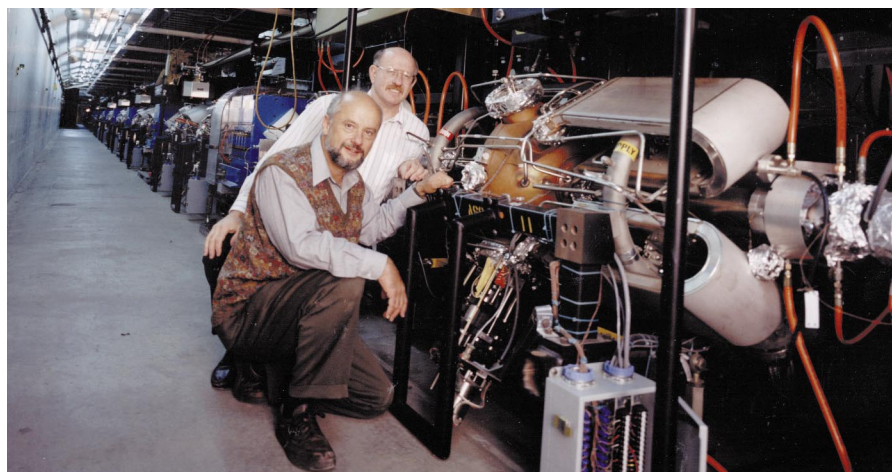
Stanford, California

The B-factory at the Stanford Linear Accelerator Center (SLAC) is poised to win an international race to quantify a critical element in the Standard Model of particle physics.

The accelerator is approaching full power faster than its designers expected. The international collaboration of physicists working on BaBar, the B-factory's detector, could obtain new estimates for this critical element — charge-parity (CP) violation — as early as the second half of this year. By then the B-factory may have generated and recorded ten million B mesons, surpassing the cumulative record of an older experiment at the Cornell Electron Storage Ring in New York state.

It is competing with another international team working at the Japanese B-factory at KEK, the particle accelerator at Tsukuba, which also began operation last summer. According to both Japanese and US officials, although the Japanese accelerator's planned luminosity is greater than SLAC's, it is so far performing less well (see opposite).

Both machines use two rings to accelerate electrons and positrons into collision at energies designed to momentarily create B mesons and anti-B mesons, their antimatter equivalent. The Japanese facility is designed to produce three times the SLAC facility's specified beam intensity of 3×10^{33} per cm^2 per second, according to physicists at the California facility, but so far it has obtained less than half the 1.4×10^{33} luminosity



Hands on: SLAC director Jonathan Dorfan, right, and Pier Oddone of Lawrence Berkeley Laboratory.

reached by SLAC last month. "They started running at about the same time, but they've had less success in debugging than we have," says Helen Quinn, a theorist at SLAC.

At a meeting last month to pay tribute to Burt Richter, who retired as the facility's director last year (see below), John O'Fallon, head of the high-energy physics division at the Department of Energy, praised SLAC's progress. "The B-factory is now turning up to world-class luminosity way before most people thought possible," he said.

"We hope to reach full luminosity this summer," says David Hitlin, a physicist at SLAC and spokesman for BaBar. "Were that

to happen, it would be unprecedented."

High luminosity in the B-factory's electron and positron rings translates into high rates of production of pairs of B mesons and anti-B mesons. By observing the comparative decay of a small fraction of these pairs, physicists will be able to estimate the extent of CP violation. This is the small but vastly important disparity between matter and antimatter that physicists believe enabled a surfeit of matter to escape annihilation at the birth of the Universe.

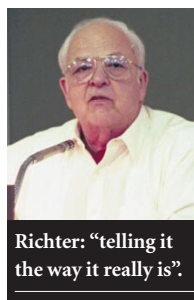
Its measurement is critical to the refinement of the Standard Model, says Quinn. "The Standard Model accommodates CP

Charm, not tact, aided pioneer in fight for physics

Stanford, California

Leaders in high-energy physics from around the world came to SLAC on 21 January to pay tribute to Burt Richter, who retired as its director last September. Richter received the Nobel Prize in Physics in 1976 for discovering the charm quark, using an experiment which he built at the laboratory before he became its second director.

Richter developed a formidable reputation for using his scientific credentials and his Brooklyn-bred persuasiveness to defend the interests of physics in general, and SLAC in particular, in Washington.



Richter: "telling it the way it really is".

celebration of Richter's career.

Early speakers recounted Richter's dogged persistence in building the SLAC Positron Electron Asymmetric Ring

"Few scientists have the ability to mediate between the conception of a problem and the practical ability to solve it," Gerhard Casper, the president of Stanford University, told participants in the day-long

(SPEAR) and its detector, and then using it to find the charm quark in November 1974. "Perhaps this was one of the few cases where you built everything from A to Z and then make all the discoveries on it," observed Haim Harari, director of Israel's Weizmann Institute.

As director of the laboratory, Richter refined the same approach, winning support for the Stanford Linear Collider (SLC) at SLAC, and then for the B-factory.

He developed considerable political clout, notably through California's large delegation to the US Congress, which became the envy of directors of other

violation and predicts relationships between CP effects. Our major aim is to find out if these relationships work or not."

Present estimates of CP violation are based on observations of another subatomic particle, the K meson or kaon, but far better measurements should be possible from studying decay channels of B mesons. Pursuit of that goal led to fierce competition between the Cornell storage ring and SLAC to build a B-factory, and to the even more ambitious Japanese project.

The time it will take to obtain results depends on what they are. If the measurement of CP violation falls in the range expected from the study of kaons, a more accurate measurement should be made quickly. If the result is smaller, it will take longer.

"This could be a hundred-yard dash, or it could be a marathon," says Jonathan Dorfan, the South African physicist who oversaw the B-factory's construction and replaced Richter as SLAC's director last September. "By the end of the year, we should have enough data to confront the issue of CP violation."

Although the BaBar detector is comparable in scale to others built in the United States, it has unusually strong non-US participation. The United States is paying \$68 million of the detector's \$110 million cost, with the rest coming from abroad, mainly from Europe. SLAC has attracted similar international participation in another major project, the Gamma-ray Large Area Space Telescope, which it hopes to build for the US space agency NASA (see box).

Dorfan admits that high-energy physicists in the United States need results from the B-factory to demonstrate that they are still making progress on big scientific questions. "The field needs some discoveries," he says.

After obtaining an initial estimate of CP violation, both B-factories are expected to study rarer B-meson decay channels (some of which occur only a few times every million events) to improve understanding of B

Sky's the limit as teams bid for NASA project

Stanford, California

Physicists at the Stanford Linear Accelerator (SLAC) are hoping to secure a vital new line of business for their laboratory next week, when the US space agency NASA is expected to announce plans for a satellite to succeed the defunct Compton Gamma Ray Observatory.

NASA will choose between rival proposals from SLAC and from its own Marshall Space Flight Centre at Huntsville, Alabama, and Washington University, St Louis, to build the main instrument for the Gamma-ray Large Area Space Telescope

(GLAST), due to be launched in 2005. "We're expecting that we'll win," says Jonathan Dorfan, SLAC's director.

SLAC's confidence is partly based on the enthusiasm professed by Dan Goldin, the administrator of NASA, for using detector technologies developed by high-energy physicists in space.

Under SLAC's proposal, NASA, the Department of Energy and a group of overseas collaborators would each carry a third of the \$120 million cost of a GLAST instrument that would use thin silicon solid-state detectors to

track gamma-ray bursts. NASA would pay an additional \$200 million to launch the spacecraft.

The project would "bring the culture of high-energy physics to work with NASA on an instrument designed and built by scientists", says David Leith, former head of the research division at SLAC. The detector would be a hundred times as sensitive as the EGRET instrument carried by the Compton observatory. The rival proposal from the Marshall Space Flight Center would use fibre-optics technology to detect the gamma-ray signals. **C.M.**

mesons and perhaps help theorists amend the Standard Model. To this end, SLAC plans upgrades that could increase the luminosity of its B-factory by an order of magnitude over five years. It hopes to make substantial progress before 2008, when a B-meson experiment is due to begin at the Large Hadron Collider at CERN, the Geneva-based European Laboratory for Particle Physics, generating a tidal wave of data on the decay of trillions of B mesons. **Colin Macilwain**

Robert Triendl adds from Tokyo: Japan's B-factory project, Belle, has reached about one-fifteenth of its projected luminosity output. Scientists at Belle say that the SLAC B-factory has achieved 1.7 to 1.8 times the luminosity of the Japanese machine, though physicists at SLAC say they are doing better than that (see above).

Several possible explanations for Belle's limited performance are being investigated, says a spokesperson for the facility, including interactions between the positron beam

and clouds of photoelectrons generated by synchrotron radiation, which may result in the beam being unfocused and blurred.

The Stanford group made efforts to deal with this problem at an early stage of the design, but observers say the Japanese design group may have underestimated the effect. Scientists at Belle say it is not yet clear why the beam is unfocused. "We are investigating various possibilities," says one. "We still think a technical breakthrough to optimize the performance of the accelerator is possible."

Several strategies to refocus the beam and optimize performance are being considered, including minimizing the photoelectron effect by replacing beam pipes and installing magnets to trap photoelectrons.

A spokesperson for Belle claims that the luminosity problems have had little effect on the scheduling of experiments. The main detectors and related data-acquisition systems are almost completed. "We hope to be able to provide some clues to CP violation by this summer," he says. ■

Former director made enemies but put SLAC on the road to success.

Department of Energy laboratories.

But Martha Krebs, assistant secretary for science at the department until late last year, praised Richter's ability to "tell it the way it really is" on behalf of all the lab directors. That role "has not been reproduced" by the other directors since Richter quit, she said.

Richter's frankness and acerbic sense of humour have made him plenty of enemies, however. Guests at the celebration included the directors of CERN (the European Laboratory for Particle Physics) and Japan's KEK, but present and past directors of Fermilab, for example, did not attend.

Some high-energy physicists think that

Richter's lack of support behind the scenes badly injured the Superconducting Super Collider project at the time of its 1993 collapse. In an interview, Richter said of the episode: "I faced a very difficult problem. As the SSC's costs went up, there was a group who said it was the SSC or nothing — they said, if that meant shutting SLAC, then shut it." It was the SSC, of course, which was closed.

Richter looks now to a new generation of leaders — Jonathan Dorfan at SLAC and Michael Witherall at Fermilab, and their counterparts abroad — to put past disagreements behind them.

"The good thing is that the new directors can blame the old ones for the problems that exist," he says.

While returning to research at SLAC, Richter is currently serving as president of the International Union of Pure and Applied Physics. He now looks to the Internet to help suspend the turf wars that adorned the second half of his career.

"We've got to get over this business of price and place," he told the physicists. "We are the inventors of the World-Wide Web, but we haven't taken advantage of it to make it irrelevant where an accelerator is built." **C.M.**

DFG leader attacks German cuts to agricultural research

Munich Ernst-Ludwig Winnacker, president of Germany's main research granting agency, the Deutsche Forschungsgemeinschaft (DFG), has criticized the German government's decision to halve its DM35 million (US\$18 million) research funding to the Ministry for Economic Cooperation (see *Nature* **402**, 845; 1999). This will mainly hit the Consultative Group for International Agricultural Research (CGIAR).

In a letter to Chancellor Gerhard Schröder, Winnacker has expressed concern that the cuts would have far-reaching consequences for developing countries, where most of the CGIAR's 16 research institutes are located. Germany, the world's largest importer of agricultural goods, has a special responsibility for supporting a high-quality system of world-wide agricultural research, he said.

Copernicus volumes stolen from Russian library

Moscow Two volumes of Nicolai Copernicus's *On the Revolutions of the Heavenly Spheres* are among 23 valuable old books stolen from

the St Petersburg Library of the Academy of Sciences (LAS). The Copernicus volumes were published in Latin in 1543 and were banned by the Catholic Church from 1616 until 1828.

Each volume is said to be worth about \$100,000. In 1998, copies of the same books disappeared from both the Polish Academy of Sciences and the Ukrainian National Library. LAS, one of the biggest book depositories in the world, stores 20 million books, including 260,000 rare volumes.

Mexican strike ends with arrest of students

Washington A nine-month strike and occupation of the National Autonomous University of Mexico (UNAM) ended without violence last Sunday (6 February) when police occupied the campus and arrested hundreds of strike leaders. The student occupation began as a protest against plans to impose tuition fees, but continued even after the university authorities had reversed their decision.

Valuing their autonomy from the government, the authorities resisted police involvement in ending the protest. But they agreed to intervention after violent incidents eroded public support for the students. With 275,000 registered students, UNAM is the largest university in the Western hemisphere and is Mexico's leading research university.

US court backs NSF plan to ditch Antarctic contractor

Irvine, California A plan by the US National Science Foundation (NSF) to switch contractors for operating US Antarctic facilities for the next ten years has been upheld by a federal court. The US Court of Federal Claims ruled that the NSF's decision to award its largest contract, worth about \$1 billion, to Raytheon of Massachusetts was appropriate, NSF general counsel Lawrence Rudolph told the National Science Board meeting last week.

The current contractor, Antarctic Support Associates of Colorado, together with another unsuccessful applicant, Brown & Root, had contested the planned award to Raytheon on procedural grounds. Raytheon will take over the contract the end of March, say NSF officials, unless the losing applicants mount a successful challenge in federal court.

UK biologists propose bioscience federation

London British biologists are calling for a new group to be created to present the interests of the biosciences to the government and the public. The Institute of Biology and the UK Life Sciences Committee — both of which already represent about a hundred specialist

interest societies — are proposing a new bioscience federation.

Consultations on the proposals are currently taking place. A new group would have a major responsibility for science policy, education and careers, and would attempt to provide an authoritative voice on public issues. A recent consultant's report to the two groups concluded that there was strong support for greater collaboration and pooling of resources.

France plans philosophy of science courses

Paris Science students in France are to learn more about the philosophy of science, the minister of research, education and technology announced last week. The government is also to create a national institute of philosophy and history of sciences, based at the Ecole Normale Supérieure in Paris. This is intended to act as a hub for research and for the creation of specialists in the philosophy of science.

From next autumn, graduate students across France enrolled in science, engineering and medical schools will be required to take course work in philosophy. Plans for the new institute will be coordinated by Dominique Lecourt, professor of philosophy at the University Denis Diderot — Paris VII.

Japan boosts industry links with universities

Tokyo In a bid to revamp Japan's industrial competitiveness, the government and the Ministry of Industrial Trade and Industry (MITI) are preparing a bill to facilitate cooperation between industry and universities. The bill will also seek to stimulate industry financing of university research, which stands at just 2.4 per cent of total expenditure.

The bill will extend universities' discretion over funds received from industry, which are currently managed by the Ministry of Education. Also, staff at public universities will be allowed to take up certain managerial positions in companies, notably board membership. The bill also includes a proposed system of reduced patent fees for universities and venture businesses.

NEAR spacecraft and Eros to have Valentine's Day tryst

Washington The next milestone for the US space agency NASA's Solar System exploration programme takes place on 14 February (St Valentine's Day), when the Near Earth Asteroid Rendezvous (NEAR) spacecraft is due to meet up with the 33-kilometre-long asteroid Eros. If all goes well, NEAR will settle into orbit around the rock for a year to study

its geology, mineralogy and chemistry.

Circling as low as 35 kilometres from the asteroid, NEAR will photograph the surface at three-metre resolution, with some images showing details as small as 10 centimetres across. A near-infrared spectrometer will map the asteroid's mineralogy with 300-metre resolution, and an X-ray/gamma-ray spectrometer will measure the abundance of key elements.

Glaxo SmithKline names senior team

London The giant merged pharmaceutical company Glaxo SmithKline has announced the names of its senior team. Jim Nidel, from GlaxoWellcome, will be the chief science and technology officer, while Tachi Yamada, from SmithKline Beecham, will head the team of senior vice-presidents leading research and development.

Both will report to chief executive Jean-Pierre Garnier. Peter Goodfellow of SmithKline Beecham will be the senior vice-president of discovery research and Allen Roses from GlaxoWellcome will be the senior vice-president of genetics research. Representatives of both companies say the issue of who set science policy is yet to be decided. Further announcements will be made once the merger is formally approved.

Will cell alliance breed bureaucracy and leave contributors out?

Sir—A recent News story reported that a US-based “multi-laboratory, multidisciplinary initiative” called the Alliance for Cellular Signalling (AFCS) seeks to provide a more integrated view of cell-signalling pathways (*Nature*, **402**, 219; 1999). To accomplish this, the AFCS hopes to “map how molecules in a cell interact” without bias towards particular proteins, through the collaborative efforts of systems engineers, biologists and informaticists. This project is to have a budget of approximately US\$100 million provided over ten years by the National Institutes of Health (NIH) and private companies.

We would like to express concern regarding both the outline of the experimental systems and the structural organization of the proposed alliance. Despite planning an integrative approach, the organization's website (<http://afcs.swmed.edu>) says that “the experimental efforts of the alliance will be focused on two cells that display interesting and important G-protein-regulated phenomena: the B lymphocyte ... and the cardiac myocyte”. Although these cell types are of obvious biomedical and pharmaceutical relevance, how can such a biased and limited view contribute to a more global perspective on cell signalling? Such an endeavour is structured without consideration of additional cell types, model systems and other interesting signalling phenomena not necessarily involving G-protein-mediated processes.

We are concerned that the hierarchical structure of this initiative creates an unnecessary division of labour and multiple layers of bureaucracy with too many committees and directors. After a selection process (based on “recognition of valued accomplishments”, according to the website) each member will be required to “contribute detailed, standardized, and quality-controlled information (from the literature) about their assigned molecules”. Besides defining “new molecules of interest”, the governing committees (“whose chairs and members have already been chosen”) will decide on the termination of membership based on an undefined concept of “failure to perform”.

It seems to us that this Orwellian structure minimizes the possibility of innovation and creative approaches towards a real understanding of cellular signalling. Furthermore, a ‘one-member—one protein’ paradigm monopolizes the contributions and marginalizes investigators in the field who are not members.

Members will be required not to publish their results in journals but rather deposit the data on the alliance's website in the form of a “molecule page” bearing their name. Although such an approach may be an efficient means of disseminating information, we feel that eliminating the impartial process of outside peer review is unsound and unacceptable. Moreover, it is unclear to us how the efforts of students and postdocs involved in the actual work will be acknowledged.

Finally, we are most concerned that this “new way of doing business [sic] that requires collaborators to act altruistically”, is restricted to a network of laboratories in North America. As stated, “collaborators cannot be spread across too many time zones”, because the alliance plans to hold teleconferences using Internet 2, which does not yet exist outside the United States. We find this reasoning shallow — and insulting to the international scientific community. The proposed research does not have to be communicated in real-time, and the Internet has worked very well for other large-scale collaborative efforts, notably the sequencing of genomes.

In view of these concerns we question whether the alliance, as it stands, deserves the genuine interest of the scientific community and the requested funding by the NIH, and whether it is such a “new research initiative” after all.

Stephen J. Haggarty,
Miguel Ramalho-Santos

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Bourne and Gilman reply—Haggarty and Ramalho-Santos have misunderstood the goals, organization and scientific approach of the Alliance for Cellular Signalling. We urge readers to visit the alliance website and decide for themselves whether the critics are right.

Haggarty and Ramalho-Santos say that the alliance's decision to emphasize signalling in only two kinds of mouse cells is “biased and limited”. But an intense focus (highly reproducible observations on a few systems) is essential to obtain complete sets of quantitative data for sophisticated mathematical modelling and systems analysis. Any criticism should be in the other direction: for hedging our bets by choosing two cell types rather than one. We plan to look at all signalling inputs to these cells, not just those initiated by G-protein-coupled receptors.

Our critics have failed to distinguish between participating investigators in the alliance and alliance membership. Participating investigators (currently 51 at 21 different research institutions) will

collaborate to run the scientific programmes of the alliance. Members of the B-lymphocyte and cardiac myocyte committees will prioritize work in seven alliance laboratories (not their own laboratories), and will make resultant data and analysis publicly available on the Internet so that all cell-signalling researchers can join in the effort. Our intention is to supply leads — for example the results of yeast two-hybrid screens and protein-interaction traps — for others to pursue and substantiate or discard.

A critical factor is Internet 2, which will permit real-time audio-visual communication with sharing of virtually any software application. We do not need to disseminate data in real time, but we must interact freely and regularly, as do the members of any research group.

We solicit members to act as consultants and to represent molecules by authorship of molecule pages as the core element of a signalling database. The job of authorship is the equivalent of writing and maintaining a structured review of the literature. Molecule pages can be collaborative; they will be peer reviewed and include comments and feedback. By facilitating bioinformatic searches for emergent properties of signalling networks, the standardized format of molecule pages should foster — not “minimize” — innovation and creativity. To date, we have received 180 applications for membership from residents of 15 countries, and we welcome and can accommodate many more. The list of molecules that need champions is a long one.

Conventional publication of data from the laboratories of participating investigators or members is not prohibited. The data to be placed on the Internet will be produced in dedicated alliance laboratories staffed by fully trained, full-time research scientists and technicians, not postdocs or students.

Henry R. Bourne*, Alfred G. Gilman†

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Proteins suggest form of their own database

Sir—A recent News report in *Nature*¹ mentioned a workshop that we organized at the National Institutes of Health concerning the prospects for databases that describe signal transduction pathways, and more specifically that define protein–protein interactions.

The complete genomic DNA sequence of an organism in principle yields its full coding potential, and gives the possibility of describing in a comprehensive fashion the structures and functions of proteins, and their organization into the pathways and networks that control cellular behaviour². The vast literature on these topics seems likely to grow exponentially with the refinement of tools for rapid proteomic analysis. We feel that the value of this information, and its ability to serve as the basis for modelling of cellular responses to external signals, will depend on its organization into a readily accessible electronic format.

One approach to such a database makes use of the observation that a common theme in cellular events is the assembly of proteins into complexes, through specific modular interactions³. A growing number of such interactions use domains and recognition motifs that can be readily identified by primary sequence analysis, and are therefore predictable⁴. This notion can be extended to encompass the interactions of distinct types of macromolecules with one another, and with small molecules. Although not all cellular phenomena can be described in these terms, the concept provides a useful starting point from which to organize data.

The purpose of the workshop was to explore continuing efforts to design databases of protein–protein interactions, and to solicit input as to the best way forward. We considered the creation of a centralized, freely available, public submission database as an achievable and highly desirable goal for the next generation of cellular analysis. Such an undertaking will be complex and prone to numerous pitfalls, but we believe it is an inevitable evolution of current biological databases. Furthermore, we consider it essential if we are to understand more fully how cellular function is controlled.

A transcript of the workshop will be posted on the NIGMS website. As this initiative proceeds, we will solicit broader input from the scientific community.

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Debating controversies can enhance creativity

Sir—I could not agree more with the author of your Opinion “Resolution to enhance confident creativity” (*Nature* **403**, 1; 2000) that “there will always be established and influential scientists who ... are resistant to looking beyond their long-held scientific assumptions”. I also agree that “too many of today’s creative scientists lack long-term security” and that “good but unconventional ideas are probably falling by the wayside”.

Peer review is important for ensuring the quality of published work and proposed studies. This quality check can prevent false information being disseminated and funds being wasted. But peer review can also restrict creativity. What can be done to improve the system?

With the emergence of electronic publications, which do not have to rely on a fixed format, the reviewing/citation components can now be integrated into publications. Supplemental information and hyperlinks can be added to electronically published papers to connect them with related information — a review or a follow-up research article, say. In this way, the value of the published work is automatically revealed through the reading of linked literature. Ultimately, pre-publication review might even be eliminated and replaced with continuous post-publication review, creating a free atmosphere for expressing creative ideas.

I have started such an experiment and created an electronic journal, *Logical Biology* (<http://logibio.com>), dedicated to debating controversial issues and promoting logic as a tool for scrutinizing long-held conventional views in biology: for example, the nature of bacterial life. Some people may dismiss this type of publication, but, in the interest of fostering creativity, isn’t it worth a try? The primary goal of scientific publication is, after all, not validation but communication.

Shi V. Liu

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Time for an aspirin

Sir—I found it painfully hard to read the first of the “Timescales” pages in the Impacts of Foreseeable Science supplement (*Nature* **402** (suppl.), C17; 1999). The vaguely coloured and seemingly meaningless background combined with the rather small

compressed and low-contrast typeface gave me a headache. It’s ugly, besides.

Artists should be on tap, not on top.

Marvin Minsky

MIT Media Laboratory, 20 Ames Street, Cambridge, Massachusetts 02139, USA

The myth of well-funded German research

Sir—You reported on difficulties in financing genome research in Germany (*Nature* **402**, 706; 1999). Two pages later, we read: “Lots more cash for UK universities”. These articles are symptomatic of the current funding crisis in German research. Because of the country’s past successes and its relative wealth, it is not generally known that research in Germany is no longer well funded. None of the *Länder* (states) provides enough funds to replace equipment in university laboratories — my institute has averaged less than 1 per cent of equipment costs per year for the last 20 years. Even before reunification in 1990 placed serious pressure on public funding, increases in federal support for the Deutsche Forschungsgemeinschaft (DFG) were very small.

In my field, biomedicine, research funding trails that of competing countries. In the United Kingdom, support from all sources in 2000 amounts to at least US\$24 per head of population, compared with \$15 in Germany. In the United States, biomedical funding from the National Institutes of Health (NIH) and Howard Hughes Foundation alone represents \$74 per head of population — five times higher than all such funding in Germany. Japan intends to double its science budget and the NIH has started very well on its target of doubling US biomedical research funds within five years. In the United Kingdom, funding by the private Wellcome Trust alone exceeds all equivalent funding from the DFG. Although the DFG has given out 59 per cent more for standard grants over the past ten years, this is no increase at all when corrected for inflation and increases in population.

Germany has few natural resources and relies on its best minds to produce knowledge and wealth. It needs a huge increase in public spending on research and a massive infrastructure-replacement fund from federal and state governments. It is time for scientists and learned societies to begin lobbying much more intensively: German science will only be able to compete if funding is tripled or quadrupled in the next five years.

Geoffrey A. Manley

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Worth more dead than alive

Should we attempt to conserve biodiversity simply for its own sake?

Requiem for Nature

by John Terborgh

Island Press: 1999. 656 pp. \$24.95

Harold A. Mooney

John Terborgh has written a dispiriting book. He paints a grim picture of the current status and prognosis for tropical-forest ecosystems. His bottom line is that attempts by both local and international groups to deal with the conservation of natural tropical-forest ecosystems are not working and, in some cases, are acting against the long-term viability of the few preserves that exist. Given that the only solution Terborgh suggests will seem impractical to most, or at least unachievable in the time we have to save what exists, *Requiem for Nature* leaves the reader feeling quite hopeless.

What has led Terborgh to such conclusions? He carefully lays out his credentials as a long-term observer of the status of tropical parks, particularly those in Peru but also in other parts of the tropical world. What he has seen is the steady erosion of the integrity of preserves as population pressures build up around them, coupled with a lack of institutional capacity to deal with local threats to the wildlife. Further, Terborgh has documented the large areas that are needed to maintain top predators in tropical forests, and finds that these are often much larger than the sizes of preserves. And he has shown the substantial consequences that the loss of these predators will have for the dynamics of forest ecosystems.

What does Terborgh think we should be preserving? He states that the concept of biodiversity embodied in the Convention on Biological Diversity is too broad (genes to ecosystems), and that we should focus on species. However, he then notes that it is actually species interaction (the web of interactions) that we should be preserving, which would encompass the higher-level species. He doesn't think the human species should be part of these interactions, and certainly not part of the parks.

Terborgh's strong message is that we should be conserving biodiversity for its own sake and not for any utilitarian value, and that arguments for conservation "must be spiritual and aesthetic". He makes this point to counter conservation efforts that have been based on the potential economic return to be obtained from intact forests through their exploration for medicines and other products, or the development of ecotourism. He states that these money-generating activities will be insufficient to save the tropical forests and that, to use his catchiest phrase,

these forests are "worth more dead than alive". No doubt this assertion will cause considerable consternation among conservationists. Terborgh does not evaluate, or even discuss, the concept of ecosystem services and how they might play into the conservation equation. However, it would be hard to do this within the context of the inefficient social systems that he attributes to many of the developing countries in which the tropical parks lie.

The book produces some compelling figures: 50 per cent of the world's biodiversity is contained in only seven per cent of the Earth's land surface that constitutes tropical forests. Only eight per cent of these forests are protected, at least on paper. Terborgh's point is that these park designations do not give a real sense of their lack of protection. He

notes that the area of park needed to support viable populations of some of the top predators in these systems is about one million hectares. Few parks are this large. According to conservative estimates, the last tropical old growth, apart from that in parks, will be gone by the middle of this century, making park size and integrity an even more pressing concern.

So what is Terborgh's answer? First, we must do something about the forces that are confounding conservation efforts — "overpopulation, inequities of power and wealth, exhaustion of natural resources, corruption, lawlessness, poverty and social unrest". More directly, we need to design better protection for the parks. Terborgh holds the US park service up as a model of what is needed: well-trained people who know the parks and have



A receding wilderness

Indonesia's expanding logging industry is decimating the lowland rainforests of Sumatra and Borneo (shown above). The lavishly illustrated *Archipelago: The Islands of Indonesia, From the Nineteenth-Century Discoveries of Alfred Russel Wallace to the Fate of Forests and Reefs in the Twenty-First Century* by Gavan Daws and Marty Fujita (University of California Press, \$45, £27.50) describes the

14,000-mile journey made by the naturalist Alfred Russel Wallace among these islands — it was while on the archipelago that, independently of Charles Darwin, Wallace developed a theory of evolution by natural selection. The book's final chapter looks at twentieth-century Indonesia and the ravages to biodiversity that have been wrought by the country's economic imperatives.

the authority to protect them, combined with a budget to do this and a human population that values their existence.

Terborgh believes that this model cannot be achieved with the current pressures of populations and the social systems that prevail in many of the tropical nations. He feels that stewardship and management of natural systems cannot be left in the hands of the local people in most of these regions. He proposes instead a top-down effort, a sort of UN nature-keeping force, to staff the tropical parks in those countries where there is still the possibility of saving something.

This is pretty radical stuff, and is diametrically opposed to many efforts that are taking 'the village' as the fundamental conservation unit and building upwards, using local knowledge and needs.

It is interesting to compare Terborgh's position with that of Daniel Janzen, another distinguished ecologist who has had a roughly comparable career. Both Janzen and Terborgh have dedicated much of their careers to on-the-ground study of tropical forests. Each spends part of every year engaged in research in their respective study regions. Janzen has come to the view that "humanity now owns life on Earth. It plans the world, albeit with an unintended here and an uninformed there." He is looking towards human coexistence with nature rather than human exclusion, which would, in his view, lose the very things we are trying to protect.

Janzen has asked how we can most responsibly fulfil this management charge. He has become a gardener of natural systems, working towards their restoration and nurturing their diversity. To do this, he has engaged and energized local people, working towards his view of sustainability using many constituent parts. The viewpoints of Terborgh and Janzen could not be more different in spite of their similar experience.

Terborgh has focused on a remote, wet, tropical reserve in Peru, whereas Janzen has worked most extensively in a tropical dry forest in Costa Rica, a system that has been largely transformed by human activity. These differences could certainly lead to different viewpoints. Further, not only are the ecological systems in which they work different, but, as noted by Terborgh, Costa Rica is a model of what can be done for conservation by local governments in developing countries. He holds out less hope for most other tropical countries.

Most of us will stand in the wide middle ground between the approaches of Janzen and Terborgh. It is important, however, to have the Terborgh and Janzen signposts to help us gauge how successfully we are moving forward. ■

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Population genetics revisited

Mathematics of Evolution

by Fred Hoyle

Acorn Enterprises: 1999. 163 pp. \$36

John Maynard Smith

In 1987, just 100 facsimile copies of this book were produced. The book has now been typeset and made available to a wider audience, with a new preface by the author. In it, Fred Hoyle rediscovers many of the classical results of population genetics. Although he has already read Ronald Fisher, J. B. S. Haldane, Sewall Wright and Motoo Kimura, Hoyle has clearly rediscovered some of the main results. This is apparent from some of the less familiar ideas that he reports: for example, Müller's ratchet, Orgel's catastrophe theory of ageing and, more relevant to Hoyle's main thesis, the idea that a favourable mutation is unlikely to become established in a population unless genetic recombination occurs in the face of recurrent deleterious mutations — a point made in more detail by Joel Peck.

Has Hoyle reached other correct conclusions that are also new? This question is difficult to answer, because the book carries few references explaining which of Hoyle's findings are new, and which are merely confirmations of findings by others. However, I did spend some time on one particular topic, to which Hoyle devotes a whole chapter. This is the notion of a "cost of selection" associated with the establishment of a favourable mutation, of which he says "the claims are illusions". If true, this would be important. The idea of a cost originated with Haldane, who calculated that the number of individuals that must die selectively during the establishment of a favourable mutation lies between 10 and 100 times the population size.

Kimura used this as an argument for the neutral theory of molecular evolution. In effect, he estimated the rate at which molecular changes have occurred during evolution, and argued that the total cost of bringing about such changes was greater than the population could bear. He therefore concluded that most of the changes must be selectively neutral. My own contribution to the debate was to point out that Kimura's conclusion depends on the assumption that the costs associated with different genes can be added. This is correct if the effects of genes on fitness are independent, so that fitness effects are multiplicative. If gene effects are synergistic, the total cost is much lower.

When I first read Hoyle's discussion of this topic, I wondered whether he had rediscovered my objection, but this is not so. He does not object to the additivity of costs; it is the cost of a single substitution that he regards as

an illusion. I am baffled by what he says. I can see nothing wrong with the algebra, but the conclusion that costs are an illusion does not follow from it — it is merely an assertion. I may be mistaken, and so I hope others will look at Hoyle's argument. But my attempt to find something novel and true in his book has therefore failed. Others, however, might profitably look for nuggets of gold. After all, when Manfred Eigen and Peter Schuster reinvented evolution by natural selection, they came up with the novel and important idea of an error threshold. Hoyle is a very clever and creative scientist; it would be surprising if he has laboured entirely in vain.

Above all, however, Hoyle reaches an unjustified and ridiculous conclusion. He accepts that, given genetic recombination, natural selection can produce detailed adaptation of species to their ways of life. But he argues that it cannot be responsible for the major changes required for the emergence of new orders, classes or phyla. Something else is therefore needed. This, Hoyle asserts, is the introduction of new genetic material from outer space. He is not suggesting merely that life was first seeded on Earth from space — the theory of panspermia — an idea I find unappealing but not irrational. Hoyle argues for a continuing process of introduction.

He claims that the origin of new major groups is impossible without such intervention because, "What mutations cannot do is to find improvements which demand the simultaneous change of several base pairs". Evolutionary biologists would agree that a change requiring a number of base changes, each of which is without value until all are present, cannot occur by natural selection. They have therefore concluded that the origin of major groups has been a stepwise process, with each genetic change being an advantage on its own (although they would probably mention symbiosis as an exception). If there is no stepwise path up the mountain, natural selection won't climb it. Much thought has been given to the nature of the intermediate steps.

Hoyle gives no reason why such intermediate steps could not have existed; it is merely an assertion. In his preface, it is clear that he does not actually believe it. He says his preferred scenario is that "all genes in present day organisms were here already in the metazoans that invaded the Earth 57 million years ago at the beginning of the Cambrian era, making the subsequent story of terrestrial evolution into one in which genes have been called into operation as ecologic conditions permitted them to be so". Thus, most of these genes would have remained unexpressed because they would have been useless until appropriate conditions arose. Hoyle accepts that unexpressed genes would accumulate damage, but argues that, if populations were large enough, the deleterious mutations would not be fixed. Therefore,

when conditions were right, selection plus recombination would restore the original genetic message. But this is to assume that selection can act simultaneously at many loci, which requires that each of the multitude of genetic changes should be separately advantageous. This is precisely the assumption he has rejected in order to conclude that intervention from outer space is needed.

It is sad to see such a creative mind devoted to reaching such an absurd conclusion. It might have been better for the reputation of a great scientist if this book had been left to the decent obscurity of a facsimile edition. ■

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Secrets, codes and decoders

The Code Book

by Simon Singh

Fourth Estate/Doubleday: 1999. 416 pp.
£16.99/\$24.95

Between Silk and Cyanide: A Codemaker's War 1941–1945

by Leo Marks

Free Press/HarperCollins: 1999. 622 pp.
\$27.50 (hbk)/£6.99 (pbk)

Charles H. Bennett

Cryptography, in its narrow meaning the art of secret communication, has always been the stuff of intrigue, of military campaigns and assassinations that succeed or fail depending on whether encrypted messages survive the scrutiny of eavesdroppers. Simon Singh's *The Code Book*, a popular history in the tradition of David Kahn's classic, *The Codebreakers* (Simon & Schuster, 1997), covers the subject from Julius Caesar through medieval Arab cryptanalysis, European military and diplomatic ciphers of the past few centuries, the Second World War's Enigma and Navajo Code-Talkers, to today's struggles over encryption on the Internet.

Basic techniques of cryptography and cryptanalysis are carefully explained, as are the archaeological cryptanalyses of Egyptian hieroglyphs and Minoan Linear B. The technical and historical material is enlivened by just the right amount of biographical detail, much of it gleaned from Singh's interviews with living cryptographers.

Leo Marks' *Between Silk and Cyanide* is far narrower in scope, being a memoir of the author's work between 1942 and 1945 in Britain's Special Operations Executive (SOE), in charge of communications with secret agents in occupied Europe.

An older and perhaps even more important branch of cryptography than secret communication is authentication: the art of

establishing with whom one is communicating and verifying that their messages have not been altered en route. Indeed, some of the earliest forms of writing may have originated as a side effect of an authentication device — the Sumerian bulla, a sealed clay vessel containing loose tokens representing the kinds and quantities of goods in an accompanying shipment.

Users of secret codes have often been lulled into a false sense of security, with regard to both secrecy and authentication. A famous example described by Singh is the encrypted correspondence implicating Mary Queen of Scots in Anthony Babington's plot to assassinate Queen Elizabeth I of England. Trusting the code's security, Mary used words that left no doubt of her guilt. Elizabeth's cryptanalyst Thomas Phelippes not only broke the code of the intercepted correspondence, but even tricked the plotters into naming the planned assassins by forging, in Mary's hand, an encrypted request for this damning information. Babington, believing the coded message to be from Mary, duly

replied, and all were caught and executed.

Much of *Between Silk and Cyanide* concerns the unfortunate consequences of the 'poem code' initially used by the SOE, and Marks' effort to substitute more secure codes, including the provably unbreakable 'one-time pad'. The poem code was insecure because it depended on having the agent memorize a poem. If the Germans could guess the poem, or force a captured agent to reveal it, they could read all the agent's previous messages as well as forging new ones to their liking, which would be accepted by the SOE as authentic. To guard against such forgeries, agents were told to apply certain 'security checks' to their legitimate messages, such as a deliberate pattern of misspellings. But these security checks were often forgotten, and in any case could also be obtained by interrogating the captured agent, who would have to give some answer consistent with previously intercepted transmissions. Many agents chose to carry an SOE-provided cyanide tablet to forestall such interrogation.

The improved codes that Marks eventually put into effect resided not in an agent's memory but on a piece of silk fabric concealed in their clothing or personal effects. On the silk was printed a list of random keys, each of which was to be used to encrypt only one message, then cut off and destroyed. The security check consisted essentially of a secret password, agreed on between the agent and SOE, which the agent would include in each message before encryption.

Because each message was encrypted by a different random key, the previously intercepted transmissions revealed no information about the password itself, so a captured agent would be free to lie about it without being caught in any inconsistency. The remaining unused part of the silk was, of course, also likely to be captured along with the agent, but, lacking its cut-off portions, it neither compromised the previously transmitted messages nor enabled the agent's captors to forge legitimate-seeming new messages without knowing the password.

Meanwhile, the weaknesses of poem code had contributed to a major guessing game, with the British suspecting that many agents had fallen into enemy hands, but continuing radio correspondence with them anyway to avoid alerting the Germans of their suspicions. In one instance the Germans apparently decided they wanted the British to know that a certain agent had been captured, and so staged a Morse code radio drama in which the agent, whom Marks believes had actually been captured much earlier, was heard to begin a normal Morse transmission, which then became incoherent, finally breaking off abruptly with a sound like that of a lifeless hand resting on a telegraph key.

The two authors' styles could scarcely be more different. Singh approaches each of his many topics carefully, balancing human



Ancient Egypt's cryptic hieroglyphs

This 'praenomen', or throne name, of Ramses II is probably describing him as strong, the chosen one of the sun god Re. Many more forms of writing — from cuneiform to Japanese phonetic scripts — are featured in *The Story of Writing: Alphabets, Hieroglyphs & Pictograms* by Andrew Robinson (Thames & Hudson, £9.95).

book reviews

interest with enough technical detail to make the basic cryptographic principles intelligible to a lay reader. My only regret is that he did not give more weight to authentication and other aspects of cryptography besides secrecy, for example the role of digital signatures in electronic commerce.

By contrast, Marks' technical explanations are not always intelligible, and are sometimes buried in a torrent of colourful language: "The ladies of the First Aid Nursing Yeomanry, otherwise known as the coders of Grendon, had force-fed their eight indecipherables with a diet of transposition keys and all but one of the invalids had responded to treatment. The malingerer was waiting on my desk with a curt note from the Grendon supervisor acknowledging defeat."

Nonetheless, Marks' book makes exciting reading, and is full of information and opinions one cannot find elsewhere. Much of the information in Singh's book can be found elsewhere, but not in such an accessible and enjoyable form. ■

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A compendium of Victorian culture

The Great Exhibition

by John R. Davis

Sutton: 1999. 238 pp. £20, \$36

The Great Exhibition of 1851: A Nation on Display

by Jeffrey A. Auerbach

Yale University Press: 1999. 278 pp. \$40, £25

Sophie Forgan

The Great Exhibition occupies one of those hallowed places in history as the first and most successful of its type. It certainly had the stuff of myth in its making. The troubled background of recent revolutionary unrest in Europe, the near-disaster of the architectural design rescued by Joseph Paxton's blotting-paper sketch, the fairytale 'crystal' quality of the building which gave it its name, its huge size, the overwhelming number and richness of the exhibits gathered from all corners of the globe, the unsurpassed crowds of orderly visitors, the message of peaceful internationalism and free trade preached by its supporters — all confirm the impression of Victorian Britain at the height of its power.

The material continues to provide an inexhaustible source of anecdotes: for example, the highest tender (not accepted) for servicing the refreshment rooms came from a well-known London brothel-keeper, and the exhibition provided the launching pad for Messrs Schweppes' long



A heroic national achievement, or an object lesson in taste, mostly bad?

involvement in the soft-drinks industry.

A reassessment is timely. In 1950, Charles Gibbs-Smith viewed it as a heroic national achievement; Nikolaus Pevsner saw it as an object lesson in taste, mostly bad; Asa Briggs argued in 1954 that it was a defining moment in the formation of middle-class political and economic values, although his later work in 1988 viewed it as a compendium of 'things', of Victorian material culture. Others see it as the birthplace of modern consumer culture.

Both authors here take much from previous studies, but have their own perspectives. John Davis is particularly concerned with the political and economic context in which the exhibition evolved, and the way that exhibitions could help transform people's ideas about industrialization and modernity. His analysis of the "modernizing agenda" of the organizers has a distinctly familiar feel. Jeffrey Auerbach, on the other hand, is more interested in the exhibition as a "cultural battleground", in which ideas about national identity were forged. Both argue that the exhibition could mean quite different things to different groups, and that that was an important reason for its success.

Fully half of each book is devoted to the background and preparations for the exhibition. Davis is particularly interesting about earlier French and German exhibitions, the former having an aesthetic focus and the latter a more overtly educational agenda. The road from the original idea for a national industrial exhibition to the international exhibition of the works of industry of all nations in Hyde Park was tortuous and uncertain. The shifts of aim are carefully charted as the organizers responded to the need to gain support and encompass a broader section of the nation. It was a minor miracle that the exhibition took place at all.

Each author has valuable points to make about the choice and layout of the exhibits. Large, piled-up 'trophy' exhibits in the central avenue revealed the organizers' priorities; they generally put art or colonial raw materials in the most prestigious place. Technology and moving machinery were

popular, especially working exhibits. Visitors could watch the entire process of cotton production from spinning to finished cloth. Scientific instruments were found in class X, and included electric telegraphs, microscopes, air pumps and barometers, as well as musical, horological and surgical instruments.

Both authors tally the number of medals awarded, either for workmanship (Prize medals) or for novelty of invention or application (Council medals). They conclude, as did some scientific contemporaries, that the British had few reasons for complacency. Auerbach is better informed about relevant work in scientific history, but perhaps he accepts Charles Babbage's 'decline of science' view too readily.

The arguments both before and after the exhibition about the role of scientific and technical education in industrial progress are well aired. And the book recognizes the importance of the chemist Lyon Playfair in organizing the exhibition and helping with the classification of objects. Already a protégé of Sir Robert Peel, the former Tory prime minister, he became a protégé of Prince Albert, helped by the fact that he was also a German speaker and did his research training in Germany.

Although both books tell a similar story, their emphases are different and there is surprisingly little overlap. Davis is more sure-footed in terms of the political entanglements; Auerbach has more material on what people actually thought about the exhibition. One has a real sense of how the Great Exhibition happened and what it looked like, a vast bazaar with palm trees, flowers, organ music and the clank of machinery. Both books provide a treasure trove of pictures, many of them unfamiliar. The exhibition could clearly encompass a wide variety of meanings and interests. Minor quibbles apart, it seems that, rather like the Great Exhibition itself, there is something for everyone here. ■

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Laying down the law

Every living thing obeys the rules of scaling discovered by Max Kleiber.

Vaclav Smil

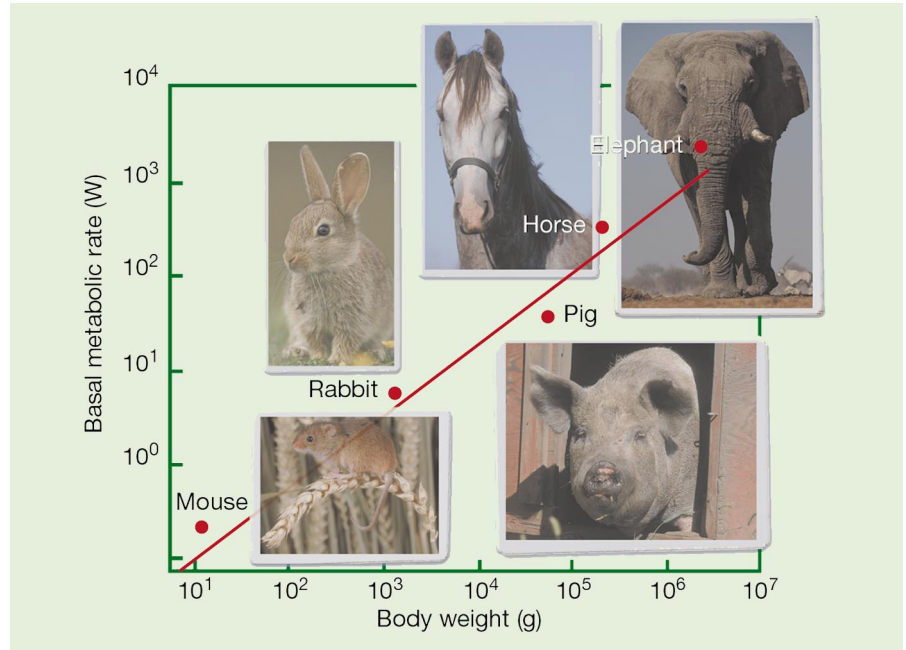
By the early 1930s, physics was a mature science abounding in universally applicable laws. In comparison, organismic biology was overwhelmingly descriptive and lacked quantitative expressions that could apply to a broad range of animals or plants. In 1932 Max Kleiber changed all that — he published a paper on “Body size and metabolism” in *Hilgardia* which included a graph plotting the log of the body weight of mammals against the log of their basal metabolic rate (BMR).

Kleiber (1893–1976) was a Swiss agricultural chemist trained at the Federal Institute of Technology in Zürich. He joined the Animal Husbandry Department at the University of California, Davis, in 1929 to study the energy metabolism of animals. Although his initial data set was rather limited, it contained mammals ranging from rats to steers — a range of body weights spanning three orders of magnitude. As BMR measures energy expenditure at rest, in a post-absorptive state (digestion increases metabolism) and in a thermoneutral environment, it conveys fundamental information about an animal's nutritional needs and allows fascinating intra- and interspecific comparisons.

Kleiber concluded that the BMR of animals depends on their body weight, w , as a function of $3.52w^{0.74}$. Kleiber's exponent differed from the traditional value of 0.67, which assumed that BMRs were a function of body surface area. Scores of species were added to the original data, extending the plot from a rat-to-steer to a mouse-to-elephant line — but the exponents of recalculated equations always came out close to 0.75.

In his 1961 book *The Fire of Life*, Kleiber opted for the 3/4 rule, recommending that BMR be expressed as $70w^{0.75}$ kilocalories per day, or $3.4w^{0.75}$ watts. As the book was widely used in university courses, often cited, and translated into German, Polish, Spanish and Japanese, the log-log mouse-to-elephant line became one of the most important and best known generalizations in bioenergetics.

Hundreds of BMRs are now available for both cold- and warm-blooded (ectothermic



Great and small: across a vast range of animal sizes, metabolic rate is proportional to $\text{weight}^{0.75}$.

and endothermic) species, and they confirm Kleiber's 3/4 law across 18 orders of magnitude, from unicellular organisms to whales. Slopes for various invertebrate groups vary from less than 0.67 to more than 1.0, but because most of them are close enough to the 0.75 line, Knut Schmidt-Nielsen has concluded that the 3/4 slope is representative for all ectotherms. While the slopes of BMRs plotted separately for various groups of organisms generally conform to the law, their positions differ substantially from values predicted by the original equation.

Predictably, the BMRs of ectotherms are only a small fraction (2.5–5%) of those of equally massive endotherms. And among endotherms, marsupials are placed about 30% lower than eutherian mammals, whereas passerine (perching) birds are placed at least 30% above non-passerine flyers. Outlying species illustrate various modes of environmental specialization. To regulate their body temperature in cold water, seals and whales have BMRs about twice as high as other animals of their size, whereas the low BMRs of desert mammals reflect their adaptation to food shortages and to recurrent or chronic scarcity of water. Not surprisingly, sloths have relatively low BMRs, but the value for pigs takes an even greater downward departure from the prediction — and hence they make efficient meat producers.

Why the 3/4 slope? In 1973, Thomas McMahon offered an explanation based on

the mechanical requirements of animal bodies. Their limb length (L) is proportional to the 2/3 power of muscle diameter (d) and as size increases L^3 is proportional to d^2 . Weight, w , of any limb is proportional to Ld^2 and hence, by substitution, w is proportional to $d^{8/3}$. Muscle cross-section appears to be the only variable determining the maximum power of a limb — also its BMR. BMR is then proportional to d^2 or to $(w^{3/8})^2 = w^{3/4}$. If it is applicable to any particular muscle, the scaling should rule the whole organism and BMR should be a function of $w^{0.75}$.

An even more fundamental explanation, offered by Geoffrey West, James Brown and Brian Enquist (*Science* **276**, 122–126; 1997), rests on the geometry and physics of the tubes that distribute resources and remove wastes in bodies. These fractal networks dictate the structural and functional properties of cardiovascular and respiratory systems in mammals and the transport of nutrients through xylem tissues in plants. Their properties require that the metabolism of organisms scales to the 3/4 power of their mass. Kleiber's law is thus extended to all life forms. In contrast, Jan Kozłowski and January Weiner have concluded that the scaling patterns across species are mere by-products of the evolutionary selection that shapes body size within species. The hunt for an explanation of the 3/4 law continues. ■

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Kleiber's law holds across 18 orders of magnitude from microbes to whales.

Only connect

An elegant demonstration that the Universe is made of quantum graphs.

Greg Egan

E. M. Forster's famous advice to 'Only connect!' is beginning to look superfluous. A theory in which the building blocks of the Universe are mathematical structures — known as graphs — that do nothing but connect has just passed its first experimental test.

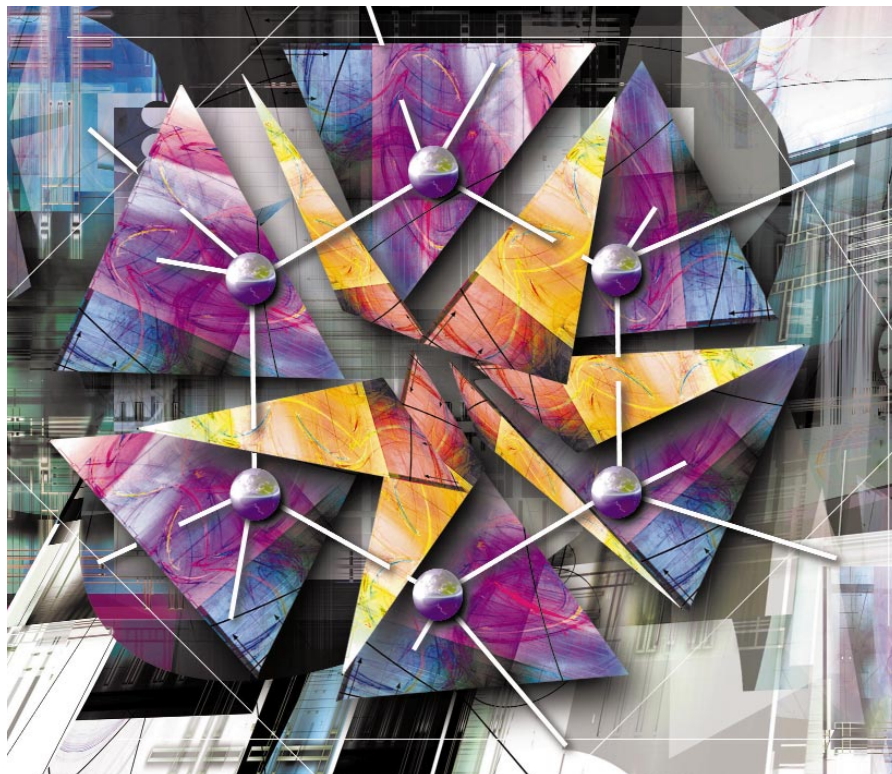
A graph can be drawn as a set of points, called nodes, and a set of lines joining the nodes, called edges. Details such as the length and shape of the edges are not part of the graph, though: the only thing that distinguishes one graph from another is the connections between the nodes. The number of edges that meet at any given node is known as its valence.

In quantum graph theory (QGT) a quantum state describing both the geometry of space and all the matter fields present is built up from combinations of graphs. The theory reached its current form in the work of the Javanese mathematician Kusnanto Sarumpaet, who published a series of six papers from 2035 to 2038 showing that both general relativity and the Standard Model of particle physics could be seen as approximations to QGT.

Sarumpaet's graphs have a fascinating lineage, dating back to Michael Faraday's notion of 'lines of force' running between electric charges, and William Thomson's theory of atoms as knotted 'vortex tubes'. More recent ancestors are Roger Penrose's spin networks, trivalent graphs with each edge labelled by a half-integer, corresponding to a possible value of the spin of a quantum particle. Penrose invented these networks in the early 1970s, and showed that the set of all directions in space could be generated from simple, combinatorial principles by imagining an exchange of spin between two parts of a large network.

Generalizations of spin networks later appeared in certain kinds of quantum field theory. Just as a wavefunction assigns an amplitude to every possible position of a particle, a spin network embedded in a region of space can be used to assign an amplitude to every possible configuration of a field. The quantum states defined in this way consist of lines of flux running along the edges of the network.

In the 1990s, Lee Smolin and Carlo Rovelli discovered an analogous result in quantum gravity, where spin-network states have a simple geometric interpretation: the area of any surface depends entirely on the edges of



Join the dots: each edge of a quantum graph carries one quantum of area.

the network that intersect it. These edges can be thought of as quantized 'flux lines of area', and in quantum gravity area and other geometric measurements they take on a discrete spectrum of possible values. It then makes sense to quantize the topology as well, with the nodes and edges of the network replacing the usual idea of space as a continuum of points.

In the first decades of the new millennium, John Baez, Fotini Markopoulou, José-Antonio Zapata and others did groundbreaking work on the possible dynamical laws for spin networks, assigning quantum amplitudes to the process of one network evolving into another. In the 2030s, Sarumpaet began to synthesize these results into a new model, based on graphs of arbitrary valence with unlabelled edges.

The geometry of three-dimensional space arises from tetravalent graphs (pictured), with the four edges emerging from each node giving area to the faces of a 'quantum tetrahedron'. Allowing graphs of higher valence runs the risk of producing an explosion of unwanted dimensions, but Sarumpaet found a simple dynamical law which always leads to the average valence stabilizing at four. However, trivalent and pentavalent nodes — which have come to be

known as 'dopant' nodes, in analogy with the impurities added to semiconductors — can persist under the Sarumpaet rules if they are arranged in special patterns: closed, possibly knotted chains of alternating valence. These loops of dopant nodes, classified by their symmetries and mutual interactions, match up perfectly with the particles of the Standard Model.

Since the area associated with the edges of a quantum graph is of the order of a few square Planck lengths, some 10^{-50} times the surface area of a hydrogen atom, it was once feared that QGT would remain untestable for centuries. However, in 2043 computer simulations identified a new class of 'polymer states': long, open chains of dopant nodes that were predicted to have energies and half-lives within the grasp of current technology to create and detect.

A search for polymer states that commenced at the Orbital Accelerator Facility in 2049 has now yielded its first success (see *J. Quant. Graphs* 15, 7895–7899; 2050). If the result can be repeated, Sarumpaet's graphs will shift from being merely the most elegant description of the Universe, to being the most likely one. ■

Greg Egan is a science fiction writer. His latest book is *Teranesia* (Gollancz).

Guilt-by-association goes global

Stephen Oliver

Clues to the function of a protein can be obtained by seeing whether it interacts with another protein of known function. This principle of guilt-by-association has now been applied to the entire protein complement of yeast.

If two proteins interact with one another, they usually participate in the same, or related, cellular functions. Yeast geneticists have a clever way of seeing whether two proteins can physically associate. They attach each of them to separate fragments of a third protein, called a transcriptional activator, which has the ability to switch on genes. If the two proteins interact, then the two fragments of the activator are reunited and a gene is switched on that produces an easily monitored colour change in the yeast cells. Because it is two hybrid proteins that are actually interacting, this method is called the two-hybrid system.

The availability of the complete genome sequence of the yeast *Saccharomyces cerevisiae*¹ raises the possibility of exploiting the two-hybrid approach to identify all possible pairwise interactions between yeast's 6,000 or so proteins. Although such an exhaustive search has been much talked about², so far only individual yeast protein complexes have been analysed in this way^{3,4}. Now, a collaborative group from the University of Washington, Seattle, and CuraGen, a biotechnology company, has taken on the daunting task of making a comprehensive two-hybrid analysis of protein interactions in yeast. The group concerned, Uetz *et al.*, report their results on page 623 of this issue⁵, and readers may manipulate the data themselves at a public web-site⁶.

Uncovering the functions of gene products predicted by the DNA sequences of entire genomes has become a sort of 'range on the 'omes'. Functional genomics involves a number of levels of investigation that have been named the genome, transcriptome, proteome and metabolome⁷ (Fig. 1). What distinguishes the last three levels from that of the genome is that they are context-dependent. The entire complement of messenger RNA molecules, proteins or metabolites in a tissue, organ or organism varies with its physiological, pathological or developmental condition. Transcriptome analysis, using DNA microarray techniques to screen large numbers of genes for messenger RNA abundance⁸, is yielding huge amounts of data about gene function. But it is an indirect approach — messenger RNAs are transmitters of genetic information, not functional cellular entities.

This is not true of proteins and metabolites, but comprehensive analyses of the

Level of analysis	Definition	Status	Method of analysis
Genome	Complete set of genes of an organism or its organelles	Context-independent (modifications to the yeast genome may be made with exquisite precision)	Systematic DNA sequencing
Transcriptome	Complete set of messenger RNA molecules present in a cell, tissue or organ	Context-dependent (the complement of messenger RNAs varies with changes in physiology, development or pathology)	Hybridization arrays ⁸ SAGE ¹³ High-throughput Northern analysis ¹⁴
Proteome	Complete set of protein molecules present in a cell, tissue or organ	Context-dependent	Two-dimensional gel electrophoresis, peptide mass fingerprinting ^{9,10} Two-hybrid analysis ⁵
Metabolome	Complete set of metabolites (low-molecular-weight intermediates) in a cell, tissue or organ	Context-dependent	Infra-red spectroscopy ¹⁵ Mass spectrometry ¹⁵ Nuclear magnetic resonance spectroscopy ¹⁶

Figure 1 Levels of gene-function analysis. The work of Uetz *et al.*⁵, discussed here, applies two-hybrid analysis to the yeast proteome. Details of the methods used are to be found in the references cited; SAGE is serial analysis of gene expression.

proteome and metabolome are more technically demanding. Moreover, 'classical' proteomics, involving the techniques of two-dimensional gel electrophoresis (for protein separation) and peptide mass fingerprinting (for protein identification)^{9,10}, discards information of immense value in making functional assignments. This is because extracts are immediately placed in denaturing conditions, thereby destroying all protein–protein interactions. Discovering that a protein of unknown function interacts with one of known function provides a valuable clue to the role of the novel gene product, a concept that has been termed guilt-by-association. So a parallel approach to proteome analysis is to examine not the relative abundance of proteins, but their potential interactions with one another.

Two-hybrid analysis (Fig. 2, overleaf) works by separating the coding sequences for the DNA-binding and activation domains of a transcriptional activator and cloning them into separate vector molecules. The coding sequence of a candidate protein whose partners are sought (known as a 'bait') is then

fused with the DNA-binding domain. A library of coding sequences for proteins that might interact with the 'bait' (called 'prey') is made in fusion with the activation domain. Yeast (like most sensible organisms) has two sexes, called α and a . Therefore, 'baits' and 'prey' can easily be introduced into the same yeast cell by mating. If they physically interact, the DNA-binding and activation domains are closely juxtaposed and the reconstituted transcriptional activator can mediate the switching-on of the gene that effects the colour change.

Uetz *et al.*⁵ have used two different strategies in a comprehensive analysis of yeast protein–protein interactions. In array screening, 6,000 yeast colonies, each expressing a different 'prey' molecule, were distributed into microtitre trays. Strains expressing different 'bait' molecules (192 in all) were mated to each member of the array and the positive interactions were identified. One advantage of the array approach is that it rapidly becomes clear which locations produce false-positive interactions, providing reassurance that the system is working



100 YEARS AGO

The proposal to generate electricity on the Canadian side of the Niagara and to sell electric power on the American side, has caused a flutter of excitement among American electricians. The *New York Electrical Review* states that the question has been raised whether foreign-made electricity is not subject to a duty of ten per cent *ad valorem* as an "unenumerated manufactured article." This question has produced a flood of debate, and while it is purely hypothetical as yet, the Merchants' Exchange of Buffalo and the Niagara Falls Power Company, have gone so far as to pass resolutions opposing such taxation. Those who desire discrimination in favour of home-made electricity argue that electric power is a vendable and valuable product of manufacture; that it can be measured easily and accurately, and that foreign-made electricity should pay duty equally with foreign-made cloth or wine. Those who believe in free trade in electricity point out that it is not an article, it is not valuable or sold or saleable, that it has no power to do work, but only serves as a means of transmitting power, and that it is utterly impossible to import it because it instantly returns to its source.

From *Nature* 8 February 1900.

50 YEARS AGO

Throughout Scandinavia most original scientific work is now published in the English language. Some authors write in English and others have their work translated locally; but in many cases it is advisable to obtain the services of an English-speaking man of science to check the manuscript. A most desirable course would be for such material to be checked by an English-speaking specialist who is working on almost identical lines and thereby has a natural interest in the work. The British Council has, therefore, initiated a scheme whereby it will endeavour to place each manuscript which has been submitted through its offices in Scandinavia with a scientific worker in Great Britain who is likely to have an interest in the subject in question and can carry out the checking. In general, the Scandinavians are willing to pay reasonable fees for such services, and the British Council has arranged to undertake the currency conversion for payment in sterling. The Council itself will make no charge for its services.

From *Nature* 11 February 1950.

properly. In contrast, the library screening method does not keep cells expressing the 6,000 different 'prey' molecules separate. Instead, it pools them, and each of the 6,000 strains expressing a different 'bait' is mated with the pool. Hybrid cells are selected and then screened for positive interactions.

The two strategies yield different results. The array method is more efficient, which is only partly attributable to the judicious choice of 'baits'. The library approach, while benefiting from much higher throughput, has the disadvantage that cells in the 'prey' pool compete with one another during growth and mating, so selecting against cells expressing fusion products that retard either process. Thus, of the 12 'baits' that gave positive interactions with both screens, 48 possible partners were identified by the array approach, against only 14 in the library screen.

Uetz and colleagues' analysis⁵ has yielded discoveries of three types. First, interactions between proteins of known and unknown function have indeed allowed the role of the latter to be inferred. For instance, two proteins have been linked to the metabolism of arginine (the principal storage amino acid in yeast) because they can interact with ornithine aminotransferase (an enzyme required for arginine biosynthesis).

Second, hitherto unrecognized interactions have been identified between proteins

involved in the same biological process. Yeast is an important model system for the study of the cell cycle in eukaryotes (organisms, including ourselves, whose cells have a nucleus; bacterial cells, for instance, don't). Uetz *et al.* have uncovered further interactions between cell-cycle regulators, thus extending our view of the subtlety and complexity of the control of cell growth and division. Specifically, it turns out that the cyclin-dependent kinase Cks1 interacts with each of three different B-type cyclins, implying that Cks1 may allow them to regulate the activity of the Cdc28 kinase that controls the start of yeast's cell cycle.

Finally, and perhaps most notably, the screen has provided clues for seeing how individual biological events are integrated into larger cellular processes. Meiotic recombination is the process that produces new gene combinations during the production of sperm and eggs, and ensures, for instance, that each of us is a unique individual. It involves the exchange of DNA segments between chromosomes inherited from each parent in a process called crossing-over. This takes place in a structure known as the synaptonemal complex and involves the breakage and rejoining of DNA molecules. The subsequent separation of each chromosome pair (segregation) requires the action of the filaments of a structure termed the meiotic spindle.

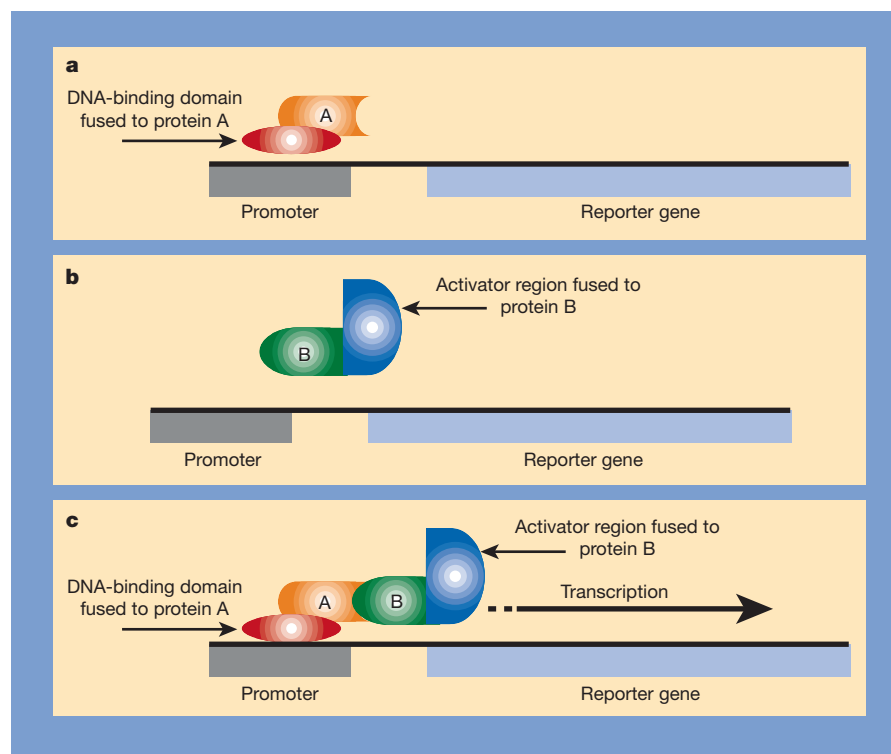


Figure 2 Detection of protein associations in yeast using the two-hybrid system. a, Fusion of the 'bait' protein and the DNA-binding domain of the transcriptional activator cannot turn on the reporter gene. b, Likewise, fusion of the 'prey' protein and the activating region of the transcriptional activator is also insufficient to switch on the reporter gene. c, When 'bait' and 'prey' associate, however, the DNA-binding domain and activator region are brought close enough together to switch on the reporter gene. The result is gene transcription and a colour change that can be monitored.

Uetz *et al.* found that Msh5 (a yeast protein required to resolve DNA cross-overs) is able to interact with two proteins implicated, respectively, in the formation of double-strand DNA breaks and the two ends of the meiotic spindle. One of these proteins, in turn, interacts with another that is required for generation of the synaptonemal complex. Thus a network of interactions is revealed that provides an overview of the mechanisms involved in meiotic recombination.

Although comprehensive two-hybrid analysis has the integrative power of all good functional genomics techniques, it lacks the context dependency of classical proteome analysis. It reveals potential protein interactions, but not the biological context in which they happen. Some may occur only when yeast is in a particular physiological state; others may never occur because, in real life, the proteins are located in separate cellular compartments.

New approaches to classical proteomics are required, which exploit separation techniques that do not destroy protein–protein interactions, and which involve methods of mass spectrometry^{11,12} and bioinformatics

that allow the unambiguous identification of all members of a protein mixture. Such a biochemical approach will complement comprehensive two-hybrid analysis. Together, the two will be a powerful way to discover the functions of newly identified proteins and integrate them into a comprehensive view of the workings of a living cell. ■

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Astronomy

Dynamics of Jupiter's atmosphere

Alvin Seiff

Understanding the complex dynamics of Jupiter's atmosphere has been difficult. The planet is mostly fluid, and in this sense is more like a star than the terrestrial planets with their thin atmospheric shells. Observed from a distance, Jupiter's atmosphere below 45° latitude is divided into dark zones and bright bands of cloud in which there are fast-moving jets and long-lived ovals (Fig. 1). The dominant oval is the Great Red Spot, a storm system that has been observed for more than 300 years. Jupiter's weather is altogether remarkably stable: the jets have been flowing east and west at nearly constant speeds for more than 100 years.

What drives this constant activity — the distant heat of the Sun or the planet's own internal energy? Sunlight, which drives the weather on Earth, is only 4% as strong on Jupiter as on Earth. Jupiter also emits almost twice as much heat as it absorbs from the Sun. The internal heat is not from the nuclear fusion that fuels a star, but is possibly generated by the heavier helium sinking into the deep interior. Solar energy should be important in the surface layers, whereas the transport of internal heat to the surface should drive activity in the deep interior. New observations^{1,2} from the Galileo Orbiter, reported on pages 628–632 of this issue, indicate that moist convection — similar to what happens

in large thunderstorms on Earth — transports significant energy upwards through Jupiter's clouds.

Moist convection has long been considered a candidate energy source for Jovian dynamics³, but there has been no firm evidence for its importance on Jupiter until now. Gierasch *et al.*¹ study visible and infrared images taken by Galileo of a region downstream of the Great Red Spot. Just before dawn they observe bright spots due to lightning, and after dawn they find two large storms at the same location. Lightning flashes have recently been seen in Jupiter's clouds⁴, but here cloud structure and depth are used to establish that lightning occurs in extremely thick, water-based clouds, up to 50 km below the surface. Such storms are reminiscent of large thunderstorm complexes on Earth, where moist convection occurs. Fluid velocities determined by tracking cloud features reveal an outflow from the storm centre, suggesting, as on Earth, upflow within the storm. Horizontal rotating eddies surround the storm, indicative of large-scale turbulence downstream of the Great Red Spot.

The authors estimate the vertical energy transport due to moist convection in the cloud. They assume that all lightning storms on Jupiter are like this one and multiply the

energy transported by the frequency of lightning storms to estimate the global rate of energy outflow. The energy transported by moist convection over the entire planet is comparable to the flux generated by Jupiter's internal heat source. This is still only a rough estimate because of the assumptions and uncertainties, but it supports the idea that moist convection can carry Jupiter's internal heat up through the cloud levels. The question is how the energy gets transported to and concentrated into these clouds.

In the other paper, Ingersoll *et al.*² integrate moist convection into their model of Jupiter's zones and belts. They expand on their earlier idea⁵ that jets in Jupiter's cloud layers are driven and maintained by momentum received from small-scale eddies. Jets usually decay into eddies, going from order to disorder in keeping with the second law of thermodynamics, as, for example, in jets emerging from aircraft engines. How Jupiter's eddies then maintain their energy is not clear. Ingersoll *et al.* now argue that moist convection is the energy source that drives turbulent eddies. But how is the vertical kinetic energy of convection converted to angular kinetic energy of the horizontal eddies? The energy is there, but what is the process?

A point not addressed in these papers is the increased wind speed below Jupiter's surface, as observed by the Galileo probe⁶. Wind speed increased from 100 m s⁻¹ at the cloud tops to ~180 m s⁻¹ at depths greater than 70 km, below which they are constant down to 150 km. This vigorous interior flow would not appear to result from cloud convection,

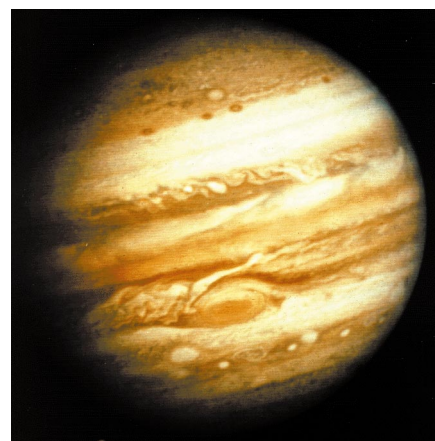


Figure 1 Image of Jupiter taken by Voyager in 1979. The dark and bright bands of clouds on Jupiter and the Great Red Spot — a 300-year-old storm — are clearly visible. What drives Jupiter's turbulent weather has long been a matter of debate. Images taken by the Galileo spacecraft in orbit around Jupiter last year provide new evidence^{1,2} that moist convection — similar to what happens in large storms on Earth — is responsible for transporting energy upwards through Jupiter's clouds.

NASA

but is more likely to reflect internal circulation of the fluid planet. With these accelerating horizontal winds, reasonable upflows ($\sim 1 \text{ m s}^{-1}$) are no longer simply vertical but are highly sheared by the wind and, relative to the cloud tops, are close to horizontal.

A remaining uncertainty is what circulation is like below the surface. Because deep observations are presently impossible, models provide the only available information. Busse proposed a model 25 years ago⁷ that was partly theoretical (from stellar circulation theory) and partly empirical. He argued that Jupiter's internal flow in the presence of a rapid rotation and an internal heat source would take the form of a series of concentric cylindrical shells aligned with the planet's spin axis. Each shell contains cylindrical vortices, having the same diameter as the shell thickness, which transport heat to the surface. According to this model, belts and zones appear where the shells intersect the surface, and they disappear at latitudes above 45° because the solid planet interior limits the radius of the innermost shell. (Photographs

at these latitudes show disorganized clouds in which lightning flashes are frequently seen.)

Busse's model was supported by experimental observations of fluid inside a spinning sphere, in which internal vortices were visible. But because observations on Jupiter are largely limited to the cloud tops, the deep flow in the interior remains very uncertain. The two new papers^{1,2} represent a notable advance in our knowledge of Jupiter's weather by demonstrating that moist convection occurs and transports significant amounts of energy. But much work remains for us to gain a broad description of Jupiter's fluid behaviour. ■

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Neuroscience

Images of lost sleep

Jim Horne

When people are deprived of sleep, the traditional view is that this causes a general reduction in arousal, affecting the entire cerebral cortex and, potentially, most aspects of behaviour. Although interesting and stimulating activities can mask this sleepiness, monotony and boredom make it worse — that's why tedious or dull types of cognitive test, such as prolonged reaction-time tasks, are particularly sensitive to sleep loss¹. Nevertheless, if a sleepy person is encouraged to try harder then performance can return to normal, at least for a while.

At first glance, the task used by Drummond *et al.*², reported on page 655 of this issue, might seem to be insensitive to sleep deprivation owing to its short duration and intrinsic interest for the sleepy subject — the authors asked subjects to memorize a verbal list of nouns. However, Drummond and colleagues begin their report by adopting a different line of reasoning³. They argue that if sleep provides the cortex with some special form of rest, then those cortical areas that work the hardest during wakefulness might have a greater need for sleep. This could leave these areas and the cognitive activities that they control more vulnerable to sleep deprivation, even with a compensatory effort of one sort or another. The authors found that this is, indeed, the case — but with an unexpected twist.

The prefrontal cortex is the region under scrutiny. Making up about 30% of the cortex, it is one of the most metabolically active areas during wakefulness. Functions of the prefrontal cortex include updating working memory, planning, knowing when and what to attend to and what to ignore, the 'sense' of time, dealing with novelty, innovation and other 'divergent' behaviours, and, of particular relevance here, aspects of verbal fluency.

Whereas previous findings⁴ have shown that sleep deprivation for 36 hours impairs the performance of even highly motivated subjects in short verbal tests, Drummond and colleagues² are the first to examine this with brain-imaging techniques. They used a non-invasive measurement of regional cerebral activity known as blood oxygen level-dependent (BOLD) functional magnetic resonance imaging (fMRI) to study the effects of about 35 hours of sleep deprivation on the responses of the prefrontal cortex to a verbal learning task. They presumed that, after sleep deprivation, the prefrontal cortex would be activated less than normal by cognitive tests that depend on this region.

The authors scanned the brains of 13 healthy young adults during verbal-learning tests done late in the afternoon, after subjects had experienced a night of either normal sleep or no sleep. High-resolution image

slices of the whole brain then enabled Drummond *et al.* to identify regions that were activated in response to the task. As might be expected, after normal sleep, areas in the left prefrontal cortex, the premotor area and the temporal lobe were specifically activated. After sleep deprivation, verbal performance (free recall) fell by about half, and BOLD activity in the temporal area was reduced. Unexpectedly, however, the areas of the prefrontal cortex that had been activated after normal sleep were activated even more after sleep deprivation. Moreover, other regions that had not been specifically activated under normal conditions (the bilateral parietal lobes and two additional areas in the prefrontal cortex) became active.

Drummond *et al.* point out that these newly activated regions were typically switched on by tasks with high working memory⁵ or cognitive loads (for example, when following a conversation, looking at a map and remembering where to go, or memorizing a new telephone number while dialling). So the authors propose that cortical regions not normally involved with the task in hand can somehow be recruited to 'help out', unless they are otherwise preoccupied. Moreover, the greater the activation in the parietal lobes after sleep deprivation, the greater the subjective feelings of sleepiness and the less the impairment to free recall.

These results led the authors to speculate, albeit cautiously, that the amount of sleepiness may indicate the degree of compensation for the sleep-deprived prefrontal cortex. They also suggest that endogenous sleep-promoting substances (such as adenosine) might be linked to both phenomena. However, these substances may represent only a control mechanism for sleep and, as such, do not necessarily explain the underlying functions of sleep.

Other tasks, such as complex and lengthy (but interesting) critical reasoning and other logical, 'convergent', rule-based tasks, are not so reliant on the prefrontal cortex. Coincidentally, such tasks are more resilient to sleep deprivation⁶ than that used by Drummond and colleagues. The authors' verbal task lasted only six minutes and consisted of nine consecutive 40-second blocks. Each comprised a short baseline period of looking at five words (this controlled for the effect of just seeing words) and then an even shorter experimental period of seeing and remembering five other words. Yet, despite the increases in BOLD (indicating activation of the prefrontal cortex), free recall nonetheless deteriorated. Drummond *et al.* point out that, had the task lasted longer, the presumed effectiveness of the compensatory responses may have declined, leading to further impairment of performance in the test. This further highlights the sensitivity of their test to sleep deprivation — and goes against the traditional view that tasks sensitive

to sleep deprivation should be monotonous and dull⁷.

Many organs in the body can rest and recover during relaxed wakefulness — to a similar extent to that achieved during sleep — but the cerebral cortex seems unable to do this. Even when we lie relaxed but awake in a dark, silent room, the cortex remains in 'quiet readiness', prepared to respond immediately. Only sleep seems to provide real rest for the cortex. This 'rest' is indicated by the characteristically large, slow (delta) waves seen in the electroencephalogram of deep, non-dreaming sleep. As Drummond *et al.* point out, this delta activity is most intense in the prefrontal cortex⁸ when there is also a particularly low rate of blood flow in this area⁹.

What really happens in the human cortex during sleep is still a mystery. Although much work has been done in rats, there is little sign of brain pathology, even when the animals are sleep deprived until death¹⁰.

However, we must bear in mind that rats have a relatively poorly developed prefrontal cortex, so the function of sleep for much of the human cortex may differ from the function of sleep in the rat.

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screening were used to increase the thermal stability⁶ and alter the preferred mechanistic pathway⁷ of several enzymes (reviewed in ref. 8). But these studies have focused on improving or altering existing properties rather than on the creation of totally new activities or the transmutation of one enzyme activity into another. To accomplish that, Altamirano *et al.*¹ have looked at nature's methods for generating novel protein functions.

It has been suggested that new enzyme activities evolve primarily by the recruitment and modification of pre-existing enzymes that catalyse a similar basic chemical step⁹. Some low level of specificity for the new substrate must exist in the target gene product, and evolution will improve the selectivity. There are many examples of this mechanism. It is also possible that certain steps in some metabolic pathways evolved by recruiting the enzyme that produced the substrate, or used the product of the new reaction to be catalysed — this route ensures specificity but the chemistry must evolve¹⁰. Examples of this mechanism are not common, but there is one dramatic case: in a number of metabolic pathways, two consecutive, but different, transformations are catalysed by proteins with similar three-dimensional structures. These enzymes all have the so-called TIM-barrel fold, named after the structure of the glycolytic enzyme, triosephosphate isomerase, in which it was first discovered¹¹ (Fig. 1).

The TIM barrel is perhaps the most common polypeptide-chain fold in biology — about 10% of all enzymes have at least one domain that adopts this structure. The fold is an especially robust one, tolerating loop replacement¹² and even the insertion of entire domains into the loop regions without disruption. It also has another characteristic that drew the attention of Altamirano *et al.* The residues that bind the substrate and so determine specificity are for the most part contributed by the barrel itself, whereas the residues that carry out the chemical transformation are located primarily in the loops that connect the helices and β -strands (Fig. 1). So the TIM-barrel fold may represent an ideal scaffold for the creation of new enzyme activities because the chemistry catalysed by the protein can perhaps be altered without loss of specificity. To demonstrate this, Altamirano *et al.* set out to convert indole glycerol phosphate synthase (IGPS) into phosphoribosyl anthranilate isomerase (PRAI).

To be fair, their chosen target has a large bull's-eye. PRAI and IGPS catalyse consecutive reactions in the biosynthesis of tryptophan. The product of the PRAI reaction is the substrate for IGPS, although no cross-reaction exists between the two natural enzymes, and their active sites differ considerably. With the aid of the known crystal structures of the two enzymes, Altamirano

Enzyme evolution

Design by necessity

Gregory A. Petsko

"The designs of his bright imagination were never etched by the sharp fumes of necessity", commented the English poet Francis Thompson in the *Dublin Review* of 1908. The poetry of Percy Bysshe Shelley may indeed, as Thompson concluded, have been designed in the fire of his imagination. Protein structure, on the other hand, is etched entirely by the sharp fumes of necessity, through the process of natural selection. On page 617 of this issue, Altamirano *et al.*¹ show that a combination of design and necessity can rapidly evolve one enzyme in a biosynthetic pathway into another. As well as providing a possible model for the natural evolution of new enzyme activities from an existing scaffold, the success of this procedure suggests that the particular scaffold they use may have advantages in the directed evolution of new biocatalysts.

The first efforts to design proteins *de novo* aimed to create sequences, which adopt a particular fold that is stable at ordinary temperatures^{2,3}. Parallel efforts to engineer new functions into natural proteins began by altering specificity using site-directed mutagenesis⁴. Despite some success with both, the creation of totally new enzyme activity by either of these 'rational' approaches has proven to be extremely difficult. No surprise, then, that the latest attempts have borrowed a leaf or two from nature's book.

In an early example of what has been termed the 'directed evolution' approach, Blacklow *et al.*⁵ subjected a sluggish mutant

of the enzyme triosephosphate isomerase to random mutagenesis followed by selection for improved catalytic potency. A number of substitutions at sites other than that of the original mutation were found, suggesting that there are several solutions to the problem of increasing efficiency. Multiple cycles of random mutagenesis, recombination and

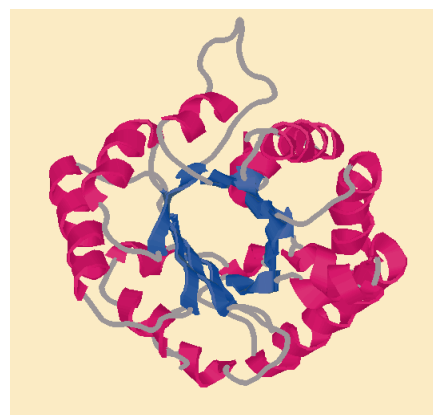


Figure 1 The folded polypeptide chain of triosephosphate isomerase, the archetypal TIM-barrel enzyme. There are eight α -helices (pink) and eight parallel β -strands (blue) in an alternating pattern. The first and eighth β -strands hydrogen-bond to each other, creating a cylinder; the α -helices line the outside of this barrel. The connecting loops are grey. The active site of TIM — and all TIM-barrel enzymes — is in the mouth of the barrel. Using directed evolution, Altamirano *et al.*¹ have converted one TIM-barrel enzyme into another with a different function.

et al. identified two active-site loops in IGPS that differed most from those in PRAI and so were targeted for replacement. Deletion of one loop followed by insertion of a shorter, random-loop library produced an ensemble of altered enzymes. The second loops of these enzymes were also modified. The mixture of mutant IGPS enzymes was then selected for PRAI activity by using them to transform a PRAI-deficient strain of *Escherichia coli* that does not grow in the absence of tryptophan. Around 500 out of 30,000 transformants were able to grow in low concentrations of tryptophan.

The second round of selection was designed to improve the function of these proteins. It involved *in vitro* sexual recombination¹³ with pools of genes from the clones selected above. Eighty colonies out of 400,000 bacteria were found to grow on very low concentrations of tryptophan; one of them could grow in its absence. Another round of DNA shuffling among the 80 clones produced 360 colonies capable of growing without tryptophan. Sequencing 30 of these revealed that only eight different sequences had been generated. Characterization of one of these proteins showed it to be soluble, stably folded and highly active.

Interestingly, although this evolved 'PRAI' enzyme has PRAI activity six times higher than that of natural *E. coli* PRAI, it lacks its original IGPS activity entirely. The improvement over the wild-type enzyme arises primarily from better binding of the substrate phosphoribosyl anthranilate¹. The sequence of the evolved 'PRAI' does not resemble natural PRAI, and there is evidence that the substrate is not bound in precisely the same way. So, converting one TIM-barrel enzyme into another by altering the loop regions has led to a different solution to the problem of catalysing the PRAI reaction.

Experiments of this kind should provide valuable insights into the machinery of divergent evolution. They also show that, with the aid of recombination, new catalytic activities can evolve quite rapidly. Similar combinations of structure-guided and selection-driven strategies are likely to be the method of choice for new protein 'design' in the near future. One can expect production of many proteins with novel functions for applications in medicine, biotechnology, organic synthesis and bioremediation at a faster rate than we could make them by 'rational' design alone. Samuel Johnson, who died eight years before Shelley was born, would have approved. In *Rasselas* (Chapter XVI), he offered advice that would seem appropriate for would-be protein engineers: "Many things difficult to design prove easy to performance."

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Developmental genetics

A Hox by any other name

Denis Duboule

In the course of evolution, the vertebrate genome experienced several rounds of duplication. As a consequence, for each gene described in flies or in worms, three or four copies of closely related (or paralogous) counterparts are routinely found in mammals. The duplicated genes may have taken on new functions by being differently regulated, but the persistence of ancestral functions amongst duplicates also generated redundancy^{1,2}. Evidence for these principles arose from the study of the mammalian Hox family, a set of 39 developmental control genes, located in four complexes derived from duplications of a single original cluster.

On page 661 of this issue³, Greer *et al.* report the analysis of functional relationships between paralogous Hox genes. By

swapping the protein-coding sequences of two Hox genes, they show that, even though their sequences are very different and the genes have distinct biological functions, their protein products are functionally equivalent. Greer *et al.* conclude that, when it comes to Hox proteins, what matters is quantity rather than quality — a provocative statement, which may have important developmental implications.

Mammalian Hox genes come in 13 groups, most of which have three or four paralogous genes. Expression analyses of the genes in group 3, *Hoxa3*, *Hoxb3* and *Hoxd3* (Fig. 1), revealed largely overlapping transcript domains, but targeted inactivation showed that *Hoxa3* and *Hoxd3* have unique functions^{4,5}. For example, mice lacking

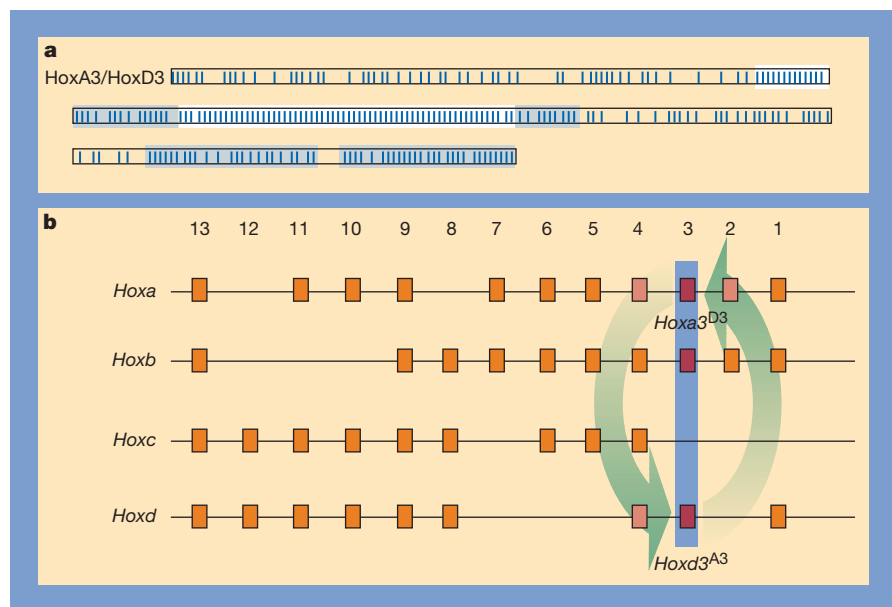


Figure 1 The *Hoxa3* and *Hoxd3* genes and their proteins. **a**, Comparison between the *HoxA3* and *HoxD3* protein sequences. Each vertical bar represents a pair of identical amino-acid residues at a conserved position. The grey boxes indicate segments of sequences that are more than 50% identical to each other. White boxes highlight the highly conserved 'pentapeptide' motif (top) and homeodomains (middle). Overall, sequence conservation is less than 50%. **b**, Scheme of the four Hox complexes with the 13 paralogous groups. Genes are indicated by the orange boxes. Group 3 is highlighted and the arrows indicate the swap between the *Hoxa3* and *Hoxd3* coding sequences carried out by Greer *et al.*³, leading to the two novel alleles *Hoxa3*^{D3} and *Hoxd3*^{A3}.

Thermodynamics

Conflicting arrows of time

Entropy is an important indicator in physics of the passage of time. Briefly, time increase is usually matched by entropy increase. Is it possible that the Universe contains regions in which time runs in a direction opposite to ours? This exciting, but unlikely, possibility has been investigated by L. S. Schulman (*Phys. Rev. Lett.* 83, 5419–5422; 1999), who has studied the entropy of two interacting gases of particles with opposing arrows of time.

If such regions exist, can each see the radiation emitted by the other? The laws of electrodynamics have also been proposed as indicators of the passage of time, because

radiation, once emitted, must be subsequently absorbed elsewhere. But electrodynamics, unlike entropy, can be shown to be time symmetric. Indeed, according to the simple cosmological equations for a model universe, such a universe may contract just as easily as it may expand. We know which solution applies to our Universe by comparison with observation.

This problem can be solved using Wheeler–Feynman absorber theory (J. A. Wheeler & R. P. Feynman *Rev. Mod. Phys.* 17, 157; 1945 & 21, 425; 1949). Suppose that electromagnetic radiation is emitted by a system, S, in both

directions of time and as equal parcels of energy. The normal radiation, R , is later absorbed by matter at S' and the remainder, r , goes backwards in time. The particles in the absorber S' are stimulated to emit radiation themselves, which also proceeds with equal energy: half (R') into the future and half (r') into the past. In due course r' reaches S, where it cancels the original wave r emitted by S into the past, leaving only the wave going forward in time, in agreement with observation.

Schulman extends this theory to his two gases with opposing arrows of time, assuming a purely theoretical interaction. He then finds that

the entropy histories of the two systems are affected by their interaction, but not as strongly as might be expected; and the results are “still consistent with electrodynamics”. On this basis, systems with opposing arrows of time may be able to coexist and see each other. Bizarre as it seems, he proposes that such objects could exist in our own Galaxy, and might have properties expected of dark matter.

Peter T. Landsberg and James Vickers

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Hoxa3 died after birth with deficiencies in the pharyngeal tissues arising from the neural crest, such as absence of the thymus⁴. In contrast, *Hoxd3* mutants survived and displayed skeletal malformations⁵. This difference in the effects of single mutations, added to a rather poor conservation between protein sequences (Fig. 1a) and similar transcript domains, suggested that the two gene products carried different qualitative values. But quantitative traits were also observed when mutations were combined: for example, vertebral defects in *Hoxd3* mutants were worsened by removing *Hoxa3* function as well^{5,6}.

To analyse these interactions in more detail, Greer *et al.*³ replaced either one of these two gene loci by its paralogous partner *in vivo*. They used homologous recombination in an embryonic stem-cell line to substitute the protein-coding region of the *Hoxa3* gene with that of *Hoxd3*, leaving the rest of the transcription unit untouched. This meant that the engineered *Hoxa3* locus contained its own regulatory DNA sequences, but produced a HoxD3 protein (the *Hoxa3*^{D3} allele; Fig. 1b). The endogenous *Hoxd3* locus was modified in the same way to produce HoxA3. These mice (for example, *Hoxa3*^{D3}) were first crossed with mice carrying the corresponding null allele (*Hoxa3*^{null}). Mouse pups with one copy of each modification (*Hoxa3*^{null}/*Hoxa3*^{D3}) survived to adulthood, indicating that one dose of HoxD3 protein was enough to compensate for the normally lethal effects of *Hoxa3* inactivation. Therefore, when expressed from the *Hoxa3* locus, HoxD3

replaced the function of the missing HoxA3. In fact, adult mice lacking HoxA3 but carrying two *Hoxa3*^{D3} alleles were indistinguishable from their wild-type littermates.

In the reverse experiment, Greer *et al.* generated mice lacking *Hoxd3* function that had either one or two copies of the *Hoxd3*^{A3} allele, so that HoxA3 was produced from the *Hoxd3* locus. Again, two copies of *Hoxd3*^{A3} completely rescued the *Hoxd3* null phenotype. But mice with only one *Hoxd3*^{A3} allele were not completely normal, suggesting that the amount of protein produced was important.

Next, Greer *et al.* produced mice that had null mutations in both copies of either the *Hoxa3* or *Hoxd3* genes, but that expressed these proteins from either the *Hoxd3* or *Hoxa3* loci, respectively. As expected, these animals (for example, *Hoxa3*^{null/null}/*Hoxd3*^{A3/A3}) displayed all the alterations associated with HoxA3 inactivation, showing that these Hox proteins, when expressed from paralogous loci, could not compensate for their endogenous inactivation. Previous experiments (for example, refs 7–9) involving gain- and loss-of-function approaches suggested that paralogous Hox genes are functionally equivalent. This elegant bidirectional complementation³ now demonstrates that a Hox protein can prevent all of the defects caused by inactivating a paralogous gene, as long as it is produced from the latter locus.

As both genes are expressed in the same patterns, why do the characteristics of the knock-out mice differ if HoxA3 and HoxD3 are functionally equivalent? One possibility

is that post-transcriptional regulation leads to variations in the distributions of the proteins. This is unlikely, though, as antibody staining for Hox proteins tends to coincide with RNA data. This implies that the overall amount of group 3 proteins is the key determinant and that modulation of this parameter induces various phenotypes. Perhaps *Hoxa3* and *Hoxd3* are differentially regulated, in quantitative terms, in different developmental contexts. Accordingly, neural crest and mesoderm cells may require particular ‘group 3 thresholds’ to initiate developmental programmes, rather than a precise qualitative pattern of gene expression. So inactivation of single genes may lead to local quantitative changes that induce different sets of morphological alterations.

It is possible that individual inactivation of either *Hoxa3* or *Hoxd3* generated defects that have not yet been uncovered, such as a metabolic or psychological problem, and that these defects were not prevented by expression of the paralogous gene. However, these results may reflect a general principle of Hox gene function whereby large-scale amplification of Hox genes early in vertebrate evolution triggered a shift in the functional strategy of their protein products. Instead of a ‘vertical’ scheme, where genes have precise and unique attributes in regulatory pathways, a ‘horizontal’ scheme may be at work, in which developmental decisions are taken by integrating quantitative inputs rather than by relying on genes’ individuality.

In the experiments described by Greer *et al.*, Hox gene duplication was unexpectedly

followed not by the emergence of novel regulatory specificities^{1,2,10}, but by an increased ability to vary local concentrations of the resulting gene products. Specific predictions can be based on these results. So further experiments in which doses of particular Hox proteins are modified in selected target organs will soon tell us whether quantity is really all that counts. ■

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Antarctica

Stirring the icy waters

Charles R. Bentley

Lake Vostok is back in the news. The report in 1994 of a giant, freshwater lake beneath four kilometres of ice in central East Antarctica¹ — one of the most remote spots on Earth — led to speculation that ancient microbes might exist in these waters, cut off from the Earth's surface for a million years or more². Because of this exciting possibility, drilling below the Russian station of Vostok over the southern end of the lake was halted 150 m above the base of the ice to prevent contamination of the lake by dirty drills and drilling fluids. The early caution was well justified — last year, bacteria were found trapped in samples of Vostok ice taken from near the base of the drill hole^{3,4}. Moreover, clear geochemical evidence⁵ showed that these ice samples had formed from frozen lake water. A report on page 643 of this issue by Siegert *et al.*⁶ confirms that ice has gradually frozen onto the base of the ice sheet from the surface of the lake in a way that encourages circulation of the lake water. This has implications for the distribution and survival of life in the lake, and for the likely age of the water.

If there is life in Lake Vostok, how long has it been isolated from the rest of the biosphere? At 280 km long and over 500 m deep in places, Lake Vostok is by far the largest of around 70 lakes found beneath the East Antarctic ice sheet⁷. There cannot have been exchange of water between these subglacial lakes and the ocean for as long as the East Antarctic ice sheet has existed in anything close to its present size — some tens of millions of years. Although the surface of Lake Vostok is slightly below sea level geographically, the pressure of the overlying ice sheet raises it hydrostatically to an equivalent height of more than 3 km above sea level. So water can have flowed only out to the ocean, not in from it, since the ice sheet formed.

Unless the material in the lake is a legacy of preglacial times — perhaps 50 million years ago — then its most likely source is the

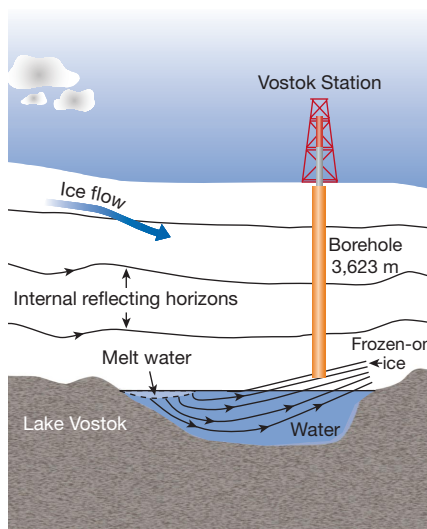


Figure 1 The East Antarctic ice sheet sliding over Lake Vostok may be concealing ancient life in the waters below. Siegert *et al.*⁶ trace radar reflections from internal layers within the ice to reveal how the ice first melts from the base of the ice sheet, then later refreezes to it. This process presumably drives water circulation in the lake, which could be important for any microbial life.

ice itself. The water that fills Lake Vostok forms as the warm Earth melts the overlying ice⁸. Basal ice that is melting now fell onto the ice sheet as snow, along with microorganisms⁹, organic carbon³ and inorganic matter⁵, half a million or more years ago¹. Presumably, the time over which material in the water has been isolated is the same as the 'age' (time since original snowfall) of the water itself. So the 'youngest' water in the lake must be at least half a million years old⁵. The age of most of the water is difficult to assess, however, because the age of specific water layers depends crucially on the deep circulation within Lake Vostok.

Rough estimates¹ suggest that it would take about 100,000 years to produce enough

water to fill the lake. Long as that time seems, it is still short compared with the age of the ice sheet, so Lake Vostok almost certainly filled long ago and present-day fluxes of water into and out of the lake are probably about equal. The age of the bulk of the lake water thus depends on circulation within the lake — does newly formed meltwater mix thoroughly into the lake, or does it simply flow across its surface and out of the downstream outlet, leaving the deeper, older water undisturbed?

The work by Siegert *et al.*^{6,10} provides a strong clue. By tracing radar reflections within the ice sheet, they demonstrate that melting of basal ice occurs where the ice sheet first flows out onto the lake; water then refreezes onto the ice sheet before it slides back onto land (Fig. 1). Presumably, this is because the melting-point temperature beneath the thicker ice near the upstream end is lower than near the downstream end, where the ice is a few hundred metres thinner. The expected rate of refreezing agrees roughly with the geochemical evidence for a 210-m layer of refrozen lake water at the downstream end⁵. Some convective mixing of the meltwater with the rest of the lake must result; as a consequence, the entire water reservoir would be exchanged on the order of every 100,000 years¹⁰.

So, the water in the lake may not be much older than the basal ice that is now melting — that is, roughly 0.5 to 1 million years old. Still, that is a long time for any microbial life living in the lake to have been isolated from the global environment. Microbial life in the lake may be based on either organic carbon or carbon dioxide, which may have originated in the overlying ice or formed in the lake itself, or both (J. C. Priscu, personal communication). In any case, the exchange of water between the ice sheet and the lake and the subsequent circulation is biologically relevant because it may result in a net transfer of gases and nutrients essential to support life in Lake Vostok⁶.

For life to exist in the lake there must be an energy source. Given the isolation of the lake from the atmosphere and ocean, the most likely source is the underlying Earth. Indeed, there is evidence that Lake Vostok sits in a tectonically active (and therefore relatively hot) rift zone¹, geologically similar to Lake Baikal or Lake Tanganyika. Life in Lake Vostok may even be a continental version of the hydrothermal-vent communities found on the spreading ridges of the ocean floor, although there is no evidence (as yet) of higher-order life forms in the lake.

Planetary geologists see Lake Vostok and its overlying ice partly as a terrestrial analogue to the putative ice-covered oceans of Jupiter's satellites Europa and Callisto, where extraterrestrial life may possibly exist. The planning of missions to these planets would benefit hugely from a natural laboratory in

which to test sterile techniques for accessing their subsurface oceans¹¹. Some environmentalists view this prospect with alarm, for fear that Lake Vostok will be contaminated during the testing process. It is my view, however, that space agencies and scientists will be just as anxious to prevent contamination as their terrestrial counterparts, and that the combined efforts of the two communities constitute the most likely path to safe and prompt access to the lake. Exciting times lie ahead as the exploration of Lake Vostok continues. ■

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Cell biology

Which way is up?

Mark Peifer and Ulrich Tepass

Building a house is a complex process. It requires detailed plans for the relative positions of roof and foundations (Fig. 1a), and a talented crew to place shingles and concrete blocks in the right places.

Imagine then the challenge faced by your body's cells. Each cell, whether skin cell or neuron, resembles a house in its polarity, with the equivalent of a roof and foundations. Further, cells are constructed from within, and each part has to be frequently renewed — constant streams of cellular shingles and concrete blocks must be shipped to the roof and foundations, respectively. The mechanisms concerned remain largely a mystery. Progress is being made, however,

and on page 676 of this issue¹ Bilder and Perrimon report the identification of a cellular architect that helps maintain cell polarity in the fruitfly *Drosophila*.

Animal cell polarity is best illustrated by cells in an epithelium, which associate side by side, creating a sheet with a defined top and bottom surface (Fig. 1b). Epithelial sheets separate one part of the body from another — for example, they line the gut, keeping food separate from the body cavity. To do this, epithelial cells place different cellular machinery on their top (apical) and on their bottom (basolateral) surfaces. For example, gut cells put glucose transporters on their apical sides facing the food, and

secrete extracellular matrix from their basal surfaces.

To understand this and similar phenomena, we need to know how cells establish polarity — what architectural plans do they follow and what structural elements are involved? And how do cells maintain polarity? Unlike a house, where a shingle nailed to the roof is unlikely to slide off, plasma-membrane proteins are in principle free to diffuse. Finally, how do cells target new proteins, the cellular shingles and foundation stones, to the correct destination?

One clue to answering these last two questions came from the realization that the apical and basolateral plasma-membrane regions are separated by structures called junctional complexes that may act as barriers. The junctional complex is typically composed of two elements: the adherens junction maintains adhesion between the two cells, while tight junctions (in chordates) or septate junctions (in other animals) act as gates preventing diffusion of extracellular molecules from one side of the sheet to the other (Fig. 1b).

Bilder and Perrimon addressed the maintenance of cell polarity by using genetic tools to look for proteins required for epithelial integrity. They worked on *Drosophila*, and so could build on earlier research that identified other key players in the process (reviewed in ref. 2). One was a protein called Crumbs, which is both a marker and a maker of 'apical character'. Crumbs is normally restricted to the apical part of the cell. In its absence, no apical region is defined; if it is overexpressed, the apical region expands. Work in *Drosophila* also revealed that, if adherens junctions are disrupted, epithelial cell polarity is compromised, supporting the

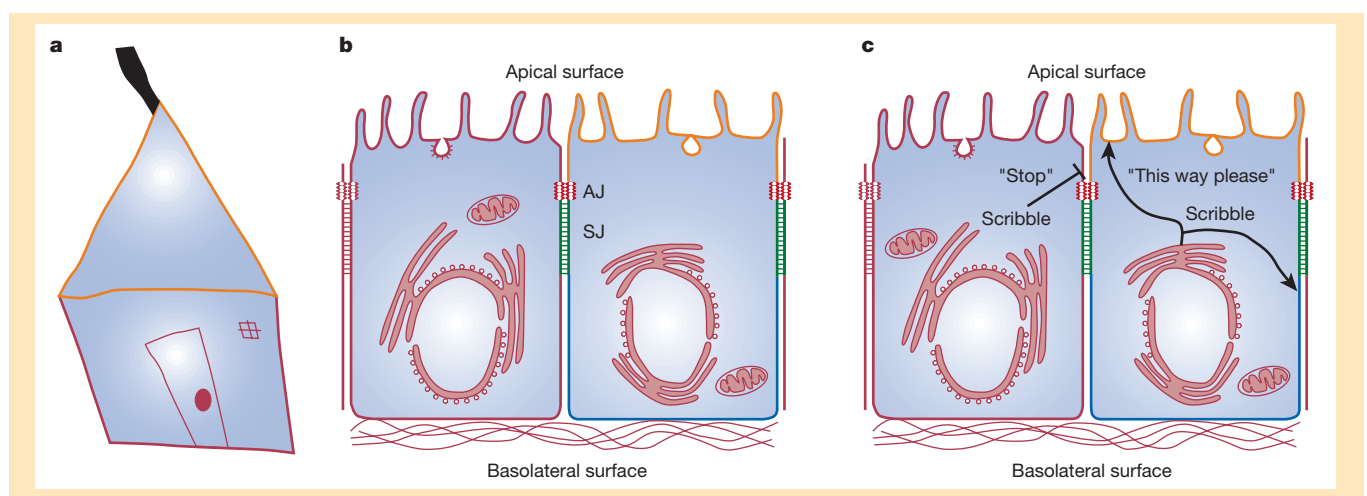


Figure 1 The architectural plans underlying home and cell construction. a, A house is polarized, with the roof (orange) always on top. b, Epithelial cells are also polarized, with separate proteins targeted to the top (apical, orange) and bottom (basolateral, blue) surfaces of each cell and thus of the epithelial sheet. Along the lateral surfaces, the junctional complex separates the two membrane domains. In *Drosophila*, the junctional complex is formed of an apical adherens junction (AJ) and a more basal septate junction (SJ; in chordates, the tight junction, which lies apical to the adherens junction). c, Two models for Scribble function are offered by Bilder and Perrimon¹ in the work discussed here. Scribble might form part of a fence preventing apical proteins from straying into basolateral territory, or it may help to direct basolateral and apical proteins to their proper locations. (Drawings by R. Peifer (a) and S. Whitfield (b, c).)

idea that this junction has a central role in cell polarity.

Bilder and Perrimon build on this base, identifying a protein called Scribble as another participant in maintaining epithelial polarity. In Scribble's absence, cells initially become polarized but, as embryo development proceeds, cell polarity is disrupted, epithelia lose their monolayered appearance, and adherens-junction proteins lose their polarized localization and become distributed around the cell periphery. In addition, apical proteins, including Crumbs, are no longer restricted to the apical surface. In contrast, septate-junction and basolateral proteins are less severely affected. So it seems that Scribble maintains the identity of the apical membrane region. As many consequences of the absence of Scribble are mimicked by deliberately mislocalizing Crumbs³, one function of Scribble may be to restrict Crumbs to the apical part of the cell.

But how does Scribble work? Bilder and Perrimon cloned the *scribble* gene, and in doing so spotlighted a family of proteins that act as molecular scaffolds on which other proteins are assembled. Scribble's protein sequence reveals a series of protein-protein interaction motifs. Its carboxy-terminal half contains four PDZ domains (named for the first three proteins in which they were found: PSD-95; the fly septate-junction protein, Discs large; and the mammalian tight-junction protein, ZO-1). A PDZ domain often recognizes specific amino-acid sequences at the carboxy termini of other proteins, but can also recognize internal protein motifs (reviewed in ref. 4). With four tandem PDZ domains, Scribble could bind four different partners simultaneously.

Moreover, Scribble's amino terminus contains leucine-rich repeats. Related repeats in other proteins bind small GTPases of the Ras superfamily. If this is true in Scribble, it might provide a mechanistic clue, as the GTPases RhoA and Rac are involved in epithelial polarity⁵ and junction function (reviewed in ref. 6).

Our knowledge of other PDZ proteins provides hints as to Scribble function. One such protein, InaD, which has five PDZ domains, brings together five proteins sequentially involved in transducing the light signal from the visual pigment rhodopsin (reviewed in ref. 7). The result is a functional signalling complex. A tripartite complex of PDZ proteins, Lin-2, Lin-7 and Lin-10, binds transmembrane proteins such as the receptor for epidermal growth factor and the glutamate receptor, mediating their targeting or retention in the basolateral region (reviewed in ref. 4). So PDZ proteins both assemble multiprotein complexes and localize them to particular cellular locations.

Many PDZ proteins are concentrated at cell junctions. The *Drosophila* PDZ proteins Canoe and ZO-1 localize to adherens

junctions⁸. Tight and septate junctions harbour many PDZ proteins, including Discs large in *Drosophila* and the ZO-1, 2 and 3 family in mammals (reviewed in refs 4, 9). Bilder and Perrimon find that Scribble also localizes to septate junctions. This contrasts with the PDZ protein Discs-large, also critical for cell polarity¹⁰, which is concentrated in the apical region. Bazooka/ASIP/Par-3, a PDZ protein essential for cell polarity in both *Drosophila* and the nematode *Caenorhabditis elegans*, is more complex: it localizes to tight junctions in mammals and the apical membrane in *Drosophila*^{11–14}.

Bilder and Perrimon propose two ways in which Scribble might work (Fig. 1c). Scribble might form part of a fence preventing apical proteins from straying into basolateral territory. Alternatively, it may help to direct basolateral and apical proteins to their proper locations.

More complex possibilities also suggest themselves. First, Scribble appears before septate junctions form, and at that time it localizes at the apical end of the lateral cell surface. So it might regulate the localization of apical proteins during this early period when it resides near them. Alternatively, if Scribble works later when it is a septate-junction protein, its effects at a distance on apical and adherens-junction proteins reinforces the idea that cell polarity results from the integrated action of proteins of the apical and basolateral regions and the junctional complex. As in home construction, if the foundations are compromised, the roof may collapse. To see which of these models best fits reality, we must identify and study Scribble's protein partners and other architects of cell polarity. ■

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Daedalus

Stirring the blood

Cardiovascular diseases are among the greatest killers of modern times. Solids precipitate out of our blood — either as dangerous deposits on arterial walls, or as clots which travel in suspension until they block some crucial capillary. Unlike human designers, nature has never invented the circulation-filter. She relies on the blood staying dependably fluid, an optimistic hope in view of the cells and solid particles densely suspended in it.

Indeed, says Daedalus, blood is only kept fluid by the continual stirring of its movement. Stagnant blood would settle out, deposit sediment, and clot. A narrowed heart valve, for example, can create a cul-de-sac of unstirred blood in the heart. Clots can form in it, and may slip out into the bloodstream with tragic consequences.

So Daedalus wants to stir our blood. He recalls the ultrasonic cleaning bath, whose intense high-frequency agitation shakes dirt off solid surfaces. Ultrasound seems biologically harmless. It is regularly used to image structures inside the body, even fetuses. Indeed, a lithotripter focuses it intensely enough to shatter kidney-stones inside the body. So stirring the blood ultrasonically should be simple and safe. The intimate agitation would break up any incipient clots and nuclei that might be forming, keeping the blood safely fluid.

DREADCO's prototype blood-stirrer is a bath equipped with piezoelectric sound-generators that launch a converging beam into the water. The patient sits in the bath. Since he is 70% water himself, the beam enters him with little refraction or reflection, and converges to the desired volume in his body. It might be the heart, or one of the wider arteries. The beam will be kept on for several minutes, until all the patient's blood has passed through the beam. Depleted of incipient clots and clumps, it will be perfectly homogenized.

Even people with very clot-prone blood can survive for years without incident. So one blood homogenization should ensure good health for a long time. But patients at high risk could be homogenized more often. They might even wear a personal ultrasound stirrer all the time. With luck, blood-stirring will have other benefits as well. Invigorated by their vibro-massage, red cells may exchange oxygen more freely, and white ones patrol the system for invaders with added vigilance. **David Jones**

The Further Inventions of Daedalus (Oxford University Press), 148 past Daedalus columns expanded and illustrated, is now on sale. Special *Nature* offer: m.curtis@nature.com

Flight restores fight in crickets

Aggressiveness recovers much faster in male crickets forced to fly after a defeat.

It is not fully understood what makes animals aggressive, although ancient Chinese gamblers, betting fortunes on the outcome of cricket fights, seemed to know a trick or two¹. Inspired by their age-old wisdom, we found that we could restore aggressiveness in defeated crickets simply by activating their motor programme for flying. This behavioural effect on aggression highlights the impact of specific motor patterns on the operation of seemingly unrelated command centres. It may also provide an insight into the mechanisms underlying motivational processes and their evolution.

Fights between male crickets follow a

stereotyped, escalating sequence. Unless one animal retreats immediately (level 1), the contestants initially fence with their antennae (level 2), and then display spread mandibles (level 3: unilateral; level 4: mutual), which later interlock (level 5) before the animals wrestle (level 6). The contest can be stopped at any level by one animal retreating. After defeat, losers avoid any more aggressive encounters (re-engagement frequency, REF, 8.2%, $n = 49$). Their readiness to fight recovers to the same level as naive, isolated males (REF 78.2%, $n = 179$) after about 24 hours (74.4%, $n = 219$).

As originally claimed by Chinese gamblers¹, defeated crickets regain their aggressiveness after being shaken in clasped hands and thrown into the air several times (REF 56.5%, $n = 23$, $P < 0.001$, χ^2 comparison with untreated losers; Fig. 1a). It proved even more effective to induce flight behaviour in a windstream. After this, 80.0% ($n = 40$, $P < 0.0001$) of the losers re-engaged the previous opponent and their aggression escalated to the same level as naive animals.

Handling, sensory stimulation, and locomotion can all cause the release of amines, which affect the aggressive behaviour of arthropods². However, the aggressiveness of subordinate crickets was not influenced by other stressful stimuli (for example, tumbling in a rotating tube), other locomotor behaviours such as 1-min episodes of wind-induced running (REF 18.2%, $n = 11$, NS), or wind stimulation when the animals had ground contact and so did not fly (REF 13.3%, $n = 15$, NS.).

The effect of flight on aggression was independent of its duration and appeared not to be transitory: a 10-s flight was as effective as a 15-min flight, and the influence of a 1-min flight was still evident hours later (Fig. 1b). The flight treatment could be

repeated many times, whenever an animal lost, without losing its effectiveness.

Aggression is commanded by the brain³, and flight is produced by a thoracic central-pattern generator⁴. When the two connectives between these centres were severed, all animals still flew, and most showed the basic elements of aggression (antennal fencing and mandible spreading). However, flying did not restore aggression in losers with their connectives cut after defeat (REF 18.8%, $n = 16$, $P < 0.001$, χ^2 comparison with intact losers after flight). Severing only one connective had no influence on the flight effect (REF 75.0%, $n = 4$, NS). This suggests that flying initiates a nervous rather than a humoral command which acts on the brain to 'reset' the aggressiveness of subordinate crickets.

The role of plasticity in behaviour and the nervous system is widely recognized^{5,6}, but it is rare for socially mediated behaviour to modify the nervous system of the performing animal — most examples occur relatively slowly, with long-term effects⁷⁻⁹. The reset of aggression by flying is the only example we know of in which activation of a specific motor pattern immediately affects an unrelated subsequent behaviour.

This phenomenon may not be unique, in which case it may stimulate some rethinking of the antidepressant effects of sleep deprivation¹⁰, and the classic ethological concept of displacement activity¹¹. Behavioural modulation of motivational aspects of brain function may yield new insight as to how and why evolutionary adaptation has connected behaviours that were previously unrelated.

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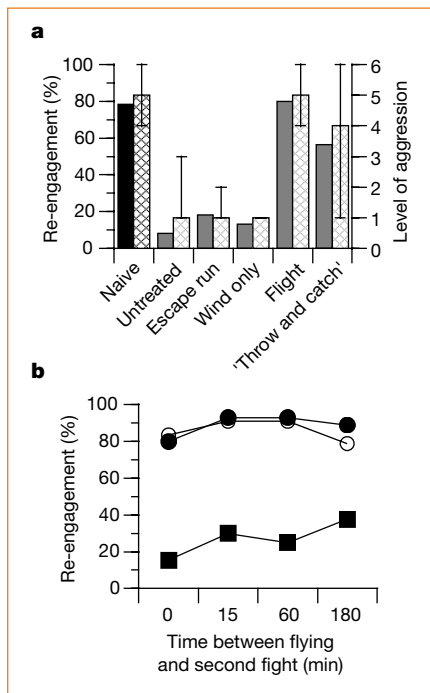


Figure 1 Behavioural depression after defeat in aggressive encounters and its alleviation by flight. Socially isolated adult male crickets, *Gryllus bimaculatus*, were placed in a 16 × 9-cm arena (details are available from the authors). Re-engagement is defined as an encounter where both animals showed overt aggression (level 4 or higher). **a**, Crickets do not fight immediately after a defeat, and losers do not re-engage after induced escape runs, or after suspension in a wind tunnel with contact to the ground (wind only). However, a 1-min flight immediately after losing restores fighting readiness in most losers, who show the same level of aggression as naive animals. Losers thrown into the air several times also re-engage significantly more often than untreated animals ('throw and catch'). Filled bars, re-engagement frequency; hatched bars, median level of aggression; error bars, interquartile ranges. **b**, Flight induces a long-term reversal of fighting readiness. Re-engagement frequency of defeated animals is independent of the time between the first fight (immediately followed by a flight) and the second fight (circles, 10-s flight; $n = 109$; filled circles, 1-min flight; $n = 88$). The re-engagement frequency of untreated crickets is shown for comparison (squares; $n = 93$).



Figure 2 Chinese gamblers inspect the merchandise in a Hong Kong shop specializing in fighting crickets.

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Physiology

The ACE gene and muscle performance

Angiotensin-converting enzyme in human skeletal muscle¹ can be encoded by either of two variants of the ACE gene², one of which carries an insertion of 287 base pairs. This longer allele gives rise to lower enzyme activity², and is associated with enhanced endurance performance³ and an anabolic response to intense exercise training⁴. Here we examine training-related changes in the mechanical efficiency of human skeletal muscle (energy used per unit power output) and find that the presence of this ACE allele confers an enhanced mechanical efficiency in trained muscle.

Subjects and staff were blind to genotype during experimentation and data analysis; genotypes are represented as 'I' for the ACE allele carrying the 287-base-pair insertion, and 'D' for the allele that does not. Caucasian army recruits (58 men: 35 II and 23 DD) were studied before and after an 11-week programme of (primarily aerobic) physical training. Subjects pedalled on a bicycle ergometer at a constant 60 r.p.m. for 3 min at each of three successive external power outputs (40, 60 and 80 W). Steady-state oxygen uptake (VO_2 in ml min^{-1}) and the respiratory exchange ratio were measured and used to calculate the energy expended per minute at each stage.

The delta efficiency (percentage ratio of the change in work performed per min to the change in energy expended per min),

which represents the efficiency of muscular contraction⁵, was calculated. We assessed changes in delta efficiency with training by using paired *t*-tests, and comparisons were made between genotype groups by independent *t*-test. Values of $P < 0.05$ were considered statistically significant.

Before training, the delta efficiency was independent of genotype (24.5 and 24.9% respectively, $P = 0.59$), but the response to training was strongly genotype-dependent, with delta efficiency rising significantly only among those of II genotype (absolute change of -0.26% for those of DD genotype ($P > 0.05$) and 1.87% for those of II genotype ($P < 0.01$): $P < 0.025$ for II compared with DD; changes were independent of all pretraining characteristics (Fig. 1)). These differences represent a proportional increase in efficiency of 8.62% relative to baseline for those of II genotype and -0.39% for DD, which may have more biological impact than this value would suggest⁶.

We do not know how the II genotype helps to improve the mechanical efficiency of trained muscle, but it may be related to an increase in slow-twitch rather than fast-twitch muscle fibres, which are more efficient in slow contraction⁷. The lower ACE enzyme activity associated with the II genotype may also raise local concentrations of nitric oxide, which in turn may improve the efficiency of mitochondrial respiration and hence contractile function in both cardiac and skeletal muscle⁸. The number of mitochondrial uncoupling proteins in skeletal and cardiac muscle drops during endurance exercise training⁹, and this reduction might be ACE-genotype dependent.

Our results have implications beyond sporting activity. The mechanical and metabolic efficiency of skeletal muscle is increased in situations in which achieving more external work for less energy utilization might be advantageous (such as lactation¹⁰ and dietary-induced energy deficiency¹¹). Congestive heart failure, for example¹², interferes with the delivery of oxygen and metabolic substrates to the whole body, so improved skeletal muscle mechanical efficiency would be an advantage. During a heart attack, the limited blood supply to the myocardium means that there is a sudden drop in the uptake of metabolic substrates and oxygen, when enhanced cardiac muscle metabolic efficiency would be an advantage.

Such benefits could be associated with lower ACE activity, an idea that may partly explain the beneficial effects of ACE inhibitors on myocardial cell survival during ischaemia¹³ and on the survival of patients with cardiac dysfunction¹⁴.

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Structural biology

A new model for protein stereospecificity

The ability of proteins to discriminate between optical isomers is vital for living systems and is exploited in drug design. Here we present a new model to explain this stereospecificity of proteins on the basis of crystallographic data.

Examples of the importance of stereospecificity are that only one of the two mirror images of the amino acid serine can activate a key receptor in the brain¹, and stereoisomers of pollutant molecules are selectively degraded in different soils².

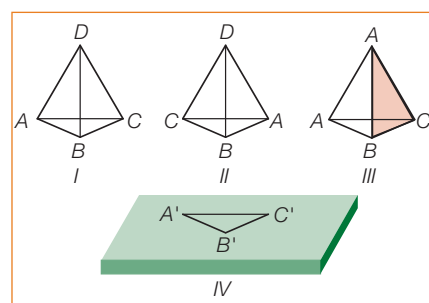


Figure 1 The original three-point attachment model^{3,8}. The ligand binds so that groups A, B, and C of one enantiomer (I) bind to protein sites A', B' and C' of the enzyme, respectively. It is easily seen that the other enantiomer (II) cannot yield an equivalent coincidence of groups A, B and C with A', B' and C'. If there are two A groups to produce a prochiral molecule (III), the model can distinguish between the two identical groups. The protein surface to which the chiral molecules bind is shown in IV.

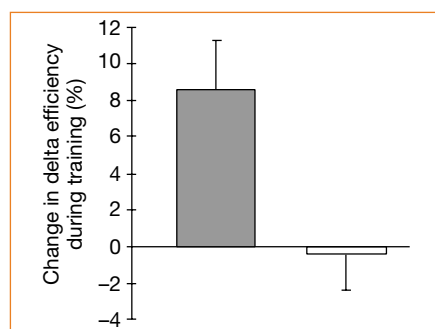


Figure 1 Change in delta efficiency (mean $\pm$ s.e.m.) during training in subjects of ACE genotypes II (shaded) and DD (white). ACE genotype was determined in 579 male army recruits as before¹⁵. At entry, 108 randomly selected homozygotes (59 II, 49 DD) exercised on an electronically braked cycle ergometer (Lode Rehor, Netherlands) at 60 r.p.m. At external power outputs of 40, 60 and 80 watts (3-min stages), the rate of energy expenditure (in watts) was calculated from oxygen uptake (ml min^{-1}) and respiratory exchange ratio (Cardiokinetics metabolic measurement cart, Medical Graphics Corp.)¹⁶. After an 11-week training period, 58 subjects (35 II: 19.4 ± 0.3 yr, 1.78 ± 0.01 m, 71.3 ± 1.2 kg; all baseline characteristics independent of genotype) were retested. Those completing the study did not differ in any measured respect from those who did not. Asterisk, $P < 0.025$. This study was approved by the appropriate ethics committee and written, informed consent was obtained from each participant.

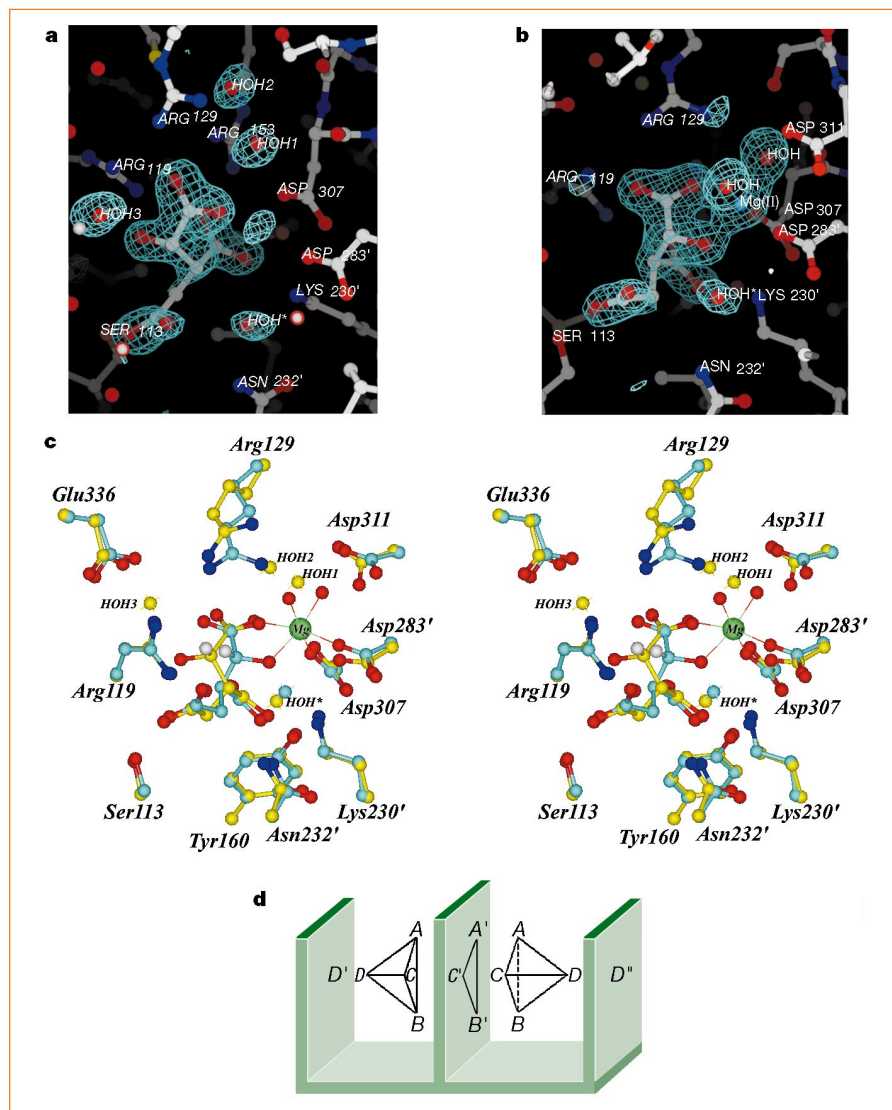


Figure 2 Protein specificity. **a,b**, Final omit maps of positive ($F_o - F_c$) difference electron density contoured at 3.0σ in the active site of complexed isocitrate dehydrogenase. **a**, L-isocitrate, and **b**, D-isocitrate plus Mg^{2+} . The structures were determined as described⁹. **c**, Stereoview of the superposition of the X-ray structures for L-isocitrate (yellow) and D-isocitrate plus Mg^{2+} (blue). Only the residues involved in enantiomeric discrimination are shown. **d**, Four-point location model for stereoselectivity of a protein, showing how a protein might provide two sites (D' and D'') in either of two locations for interaction with group D on a chiral carbon atom: D' would bind one enantiomer and D'' would bind its mirror image.

The accepted explanation^{3–6} for this stereospecificity is based on a three-point attachment model^{7,8} for enzymes and drug receptors. In this model, when three groups (A , B and C) of the tetrahedral carbon atom bind to a protein surface at specific sites A' , B' and C' (Fig. 1), it is impossible to bind the equivalent groups A , B and C of its mirror image (enantiomer) at the same three sites. We find that this model does not always hold, and use the enzyme isocitrate dehydrogenase (IDH) as an example to show that the 3-point model needs to be revised to provide a more general mechanism for stereospecificity.

When metal-free crystals of IDH are presented with a racemic mixture of isocitrate, only the L-isomer (2S,3R) binds to the enzyme, as revealed by electron-density maps of the crystal structure at 1.7 Å reso-

lution (Fig. 2a). The exclusive presence of L-isocitrate in the active site of IDH indicates that the D-isomer must bind only weakly to the metal-free (apo) enzyme. But when enzyme crystals are presented with a racemic mixture of isocitrate in the presence of Mg^{2+} , only the D-isomer is seen in the active site of the 1.85 Å crystal structure (Fig. 2b).

Superposition of the structures of the two complexes (Fig. 2c) shows that three of the four groups attached to the tetrahedral C2 atom of D- and L-isocitrate bind to the same three locations in the IDH active site. The difference is the fourth group, the hydroxyl of the C2 carbon. The -OH group of L-isocitrate associates with an arginine residue at position 119 in the metal-free enzyme, in contrast to the -OH group of D-isocitrate, which associates with the metal and with two aspartate residues at positions

283' and 307 in the active enzyme. Thus, it is the position of the fourth group, D in Fig. 1, that makes it possible for IDH to distinguish between the two enantiomers.

When this finding is generalized against published X-ray structures, the three-point attachment hypothesis only works if it is assumed that the ligand can approach a flat protein surface only from the top. In other words, a fourth location, whether a binding site or a direction, is essential to distinguish between enantiomers in an actual protein structure — so if the binding sites on the protein are in a cleft or on protruding residues, a three-point attachment will not be sufficient to discriminate between isomers. This is illustrated in Fig. 2d, where groups A , B and C of different isomers occupy the same protein locations (A' , B' and C'), whereas the D groups, which point in different directions, interact at different positions (D' and D''), as they do in IDH.

The model in Fig. 2d is called a 'four-location' model because it is not necessary for there to be four binding sites. If, for instance, there are three sites and a fourth location so that the ligand can bind in only one direction, then enantiomers can be distinguished. For example, substrates for the enzyme chymotrypsin are L-amino acids, whereas D-amino acids act as inhibitors. Our four-location model explains how D-isomers can occupy the three binding sites in an orientation that prevents cleavage. Crystal structures determined for mandelate racemase indicate that this protein also fits the four-location model.

We conclude that a four-location model is needed to explain a protein's ability to discriminate between L- and D-isomers. The four locations can, for example, be four attachment sites or three attachment sites and a direction, but a minimum of four designated locations are needed.

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Conservation biology

'Ghost' alleles of the Mauritius kestrel

The population of Mauritius kestrels is thought to have recovered from a single wild breeding pair in 1974¹, when its prospects were considered to be hopeless, to over 200 pairs today². Here we evaluate the loss of genetic variation that resulted from this bottleneck by typing 12 microsatellite DNA loci in museum skins up to 170 years old and from modern kestrels. We find that ancestral variation was remarkably high and comparable to continental kestrel species. This shows that the unexpected resilience of the population could not have been due either to benefits contributed by an undetected remnant population or to reduction of the inbreeding genetic load by a history of small population size³.

Following the widespread use of pesticides in 1940–60², the Mauritius kestrel population was for a long time smaller than the 50 individuals recommended as the minimum for viability⁴ (Fig. 1a). An intensive conservation programme between 1983 and 1993 led to a recovery to 400–500 birds⁵. Historical and post-bottleneck genotypes of Mauritius kestrels at one microsatellite locus show some of the 'ghost' alleles lost through the bottleneck (Fig. 1b). Across all loci, allelic diversity fell by 55%, and heterozygosity by 57% (Table 1). This reduction is broadly in line with the value of 0.48 expected from estimates of population size through the bottleneck⁶ (Fig. 1a).

Differences in genetic variation can be measured more precisely from estimates of effective population size, N_e . These take into account variation in genetic diversity among loci, small sample size and missing data. The likelihood of the genotypes observed at each locus was calculated as a function of $N_i\mu_j$, where N_i is population size and μ_j is the locus-specific mutation rate, which we parametrized as the differences from their means ($N_i = \bar{N} + \Delta N_i$, $\mu_j = \bar{\mu} + \Delta\mu_j$). ΔN_i and $\Delta\mu_j$ were estimated using the Metropolis–Hastings algorithm⁷.

Endemic island species show high rates of historical extinction⁸ and low genetic

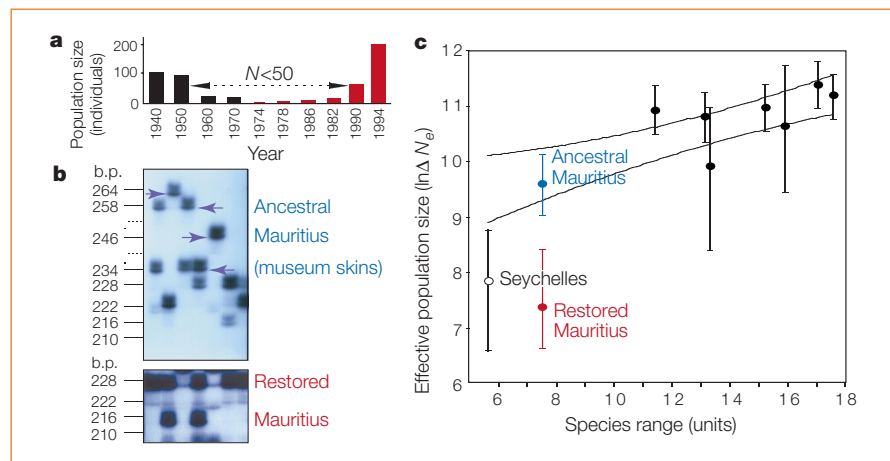


Figure 1 The genetic diversity of the ancestral population of Mauritius kestrels is similar to those of continental populations. **a**, Known population size across the bottleneck; records from Mauritius naturalists (black bars) and the Mauritius Wildlife Foundation (red bars); data calculated at 4-year intervals. **b**, Hexanucleotide microsatellite genotypes of Mauritius kestrel museum skins (top) include a mixture of alleles unique to the ancestral population (arrows) and those found in the restored population (bottom). **c**, The relationship between estimates of effective population size and species range. The 95% confidence limits are shown for the linear regression through all species, excluding the restored Mauritius (red) and Seychelles (white) population. Points show modal ΔN_e values estimated using the likelihood function for the k -alleles model; error bars show the 95% credible interval.

variation⁹. Is this low genetic diversity a cause or a consequence of their vulnerability? The relationship between our estimates of relative N_e (Table 1) and species range (current population size) shows that the ancestral N_e of the Mauritius kestrel lies within the range extrapolated from continental populations (Fig. 1c). However, both the restored Mauritius and Seychelles populations are clearly outliers. The much higher ancestral N_e on Mauritius suggests that the current low level of genetic diversity is a consequence of a recent population crisis.

Unlike some other species¹⁰, the kestrel population has recovered without the addition of new genetic variation. The kestrel population's resilience may be because its productivity was only weakly affected by the bottleneck. Laboratory studies of bottlenecked species show that the effects on productivity range from negligible to severe, and that populations may or may not recover from these in later generations¹¹.

Comparison with other tropical falcons suggests that there has been some reduction in the fitness of the Mauritius kestrel⁵. There is also evidence of an improvement since the height of the bottleneck. Direct

comparisons can only be made with the 5 years since intensive management has ceased, but productivity was consistently higher in each of these ($P=0.5^3<0.05$), averaging 1.1 fledglings per nest (31% increase). The recovery need not be a genetic response: growing populations can show surprising behavioural plasticity in colonizing habitat outside the native forest⁵.

The high ancestral level of genetic variation in the Mauritius kestrel shows that the loss of variation associated with the arrival of humans on Mauritius and the Seychelles is without recent precedent. More encouraging is that a population can recover from a bottleneck, even after a dramatic loss in genetic variation. This may help endangered island endemics stake a claim for finite conservation resources.

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Table 1 Genetic diversity of kestrel populations

Population	Sample size (2N)	Mean number of alleles	Heterozygosity*	Estimate of $\log_e \Delta N_i$
Mauritius (restored)	150*	1.41	0.10	7.38
Mauritius (ancestral)	52	3.10	0.23	9.60
Seychelles	8	1.25	0.12	7.84
European	20	5.50	0.68†	11.2
Canary Islands	16	4.41	0.64†	10.92
South African	20	5.00	0.63†	10.96
Greater kestrel	20	4.50	0.59†	10.8
Lesser kestrel	16	5.41	0.70†	11.36

*Methods for maximum likelihood estimates of heterozygosity and data on a further 225 individuals (including methods and genotypes for all populations) are available at http://www.qmw.ac.uk/~ugbt112/programs/Mauritius_kestrel/

†Significantly different from restored Mauritius population (Edwards likelihood ratio criterion, $P<0.01$).

Directed evolution of new catalytic activity using the α/β -barrel scaffold

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In biological systems, enzymes catalyse the efficient synthesis of complex molecules under benign conditions, but widespread industrial use of these biocatalysts depends crucially on the development of new enzymes with useful catalytic functions. The evolution of enzymes in biological systems often involves the acquisition of new catalytic or binding properties by an existing protein scaffold. Here we mimic this strategy using the most common fold in enzymes, the α/β -barrel, as the scaffold. By combining an existing binding site for structural elements of phosphoribosylanthranilate with a catalytic template required for isomerase activity, we are able to evolve phosphoribosylanthranilate isomerase activity from the scaffold of indole-3-glycerol-phosphate synthase. We find that targeting the catalytic template for *in vitro* mutagenesis and recombination, followed by *in vivo* selection, results in a new phosphoribosylanthranilate isomerase that has catalytic properties similar to those of the natural enzyme, with an even higher specificity constant. Our demonstration of divergent evolution and the widespread occurrence of the α/β -barrel suggest that this scaffold may be a fold of choice for the directed evolution of new biocatalysts.

Paralogs are homologous proteins that share a similar fold but have different functions. The evolution of paralogous genes is the basis for the generation of new proteins and metabolic pathways by diversification of the original function^{1–3}. Mimicking the evolution of paralogs *in vitro* would not only provide a better understanding of the natural evolution process but would also allow the development of new enzyme activities. There is considerable practical interest in new biocatalysts, with methods of accelerated or directed evolution being used increasingly in academic and industrial laboratories to modify and improve enzymes, which can then be used for the synthesis of chemicals and pharmaceuticals (for review see ref. 4). Directed evolution on a laboratory timescale requires the careful selection of a suitable starting gene. An attractive candidate for protein design and applied molecular evolution is the scaffold of the α/β -barrel class of proteins. Roughly ten per cent of all proteins belong to this structural family, which encompasses a basic motif of ~250 residues. They provide a wide spectrum of catalytic functions involving several different types of reaction^{5–9}, but have non-enzymatic roles in the cell as well.

The active site of most α/β -barrel enzymes is located at the bottom of a funnel-shaped pocket created by the loops connecting the carboxy-terminal end of the β -strands with the amino-terminal end of the α -helices that form the barrel^{10,11}. Nature has evolved the α/β -barrel to have the 'substrate-binding' residues predominantly within the barrel itself, and the 'catalytic' residues predominantly within the connecting loop regions. This approximate partition of specificity and catalysis between structurally distinct regions is a convenient device for their semi-autonomous evolution to generate diversity in a combinatorial manner. This structural feature raises the possibilities of mimicking evolution *in vitro* by grafting a new catalytic function onto an existing binding site, or a new binding site into an existing catalytic activity.

To test this procedure, we have chosen a monomeric α/β -barrel protein—the indole-3-glycerol-phosphate synthase (IGPS)—as a scaffold, and have switched its activity to that of phosphoribosylanthranilate isomerase (PRAI). IGPS and PRAI form two covalently linked domains of a bifunctional enzyme in *Escherichia coli* that

catalyses two consecutive steps in the tryptophan biosynthesis pathway¹² (Fig. 1). The enzymes have a sequence identity of 22% and share a common ligand, carboxyphenylamino-1'-deoxyribulose-5'-P (CdRP), which is the product of PRAI and the substrate of IGPS. Nevertheless, there are considerable structural differences between them: IGPS does not isomerize PRA; and PRAI does not catalyse the formation of the indole ring (Figs 1 and 2)^{13–15}. As IGPS and PRAI bind a common substrate but display different activities, they provide an excellent paradigm for testing the 'conserved scaffold' hypothesis of enzyme evolution.

Design strategy

Although there are many methods for generating diversity in a target gene^{4,16–21}, the generation of mutants must be coupled to a

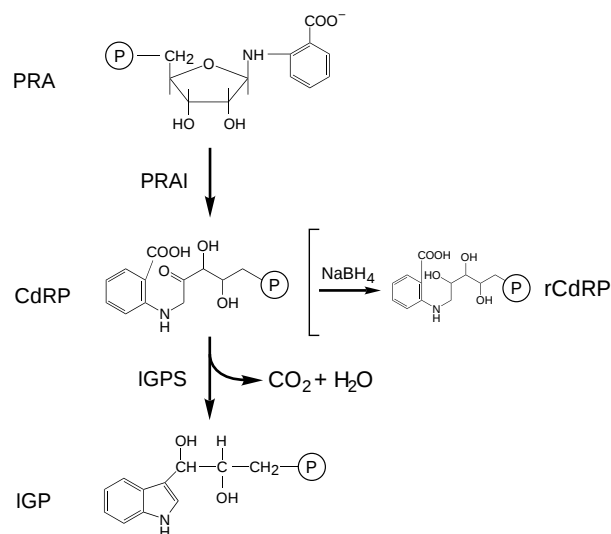


Figure 1 The reaction catalysed by phosphoribosylanthranilate isomerase (PRAI) and indoleglycerol-phosphate synthase (IGPS). The PRAI-catalysed reaction is an intramolecular oxido-reduction (Amadori rearrangement) of N-(5'-phosphoribosyl)-anthranilate (PRA) to 1'-(2'-carboxyphenylamino)-1'-deoxyribulose 5'-phosphate (CdRP). In the IGPS-catalysed reaction, the substrate CdRP undergoes an irreversible ring-closure to indoleglycerol phosphate (IGP) with release of CO₂ and H₂O. Chemical reduction of CdRP by borohydride produces the substrate analogue rCdRP; rCdRP is an inhibitor of both enzymes.

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suitable selection procedure to allow *in vitro* evolution^{4,22–25}. Moreover, as a library encoding just one copy of each possible variant for a protein of 250 amino acids (the size of the α/β -barrel) would contain 20^{250} variants, a number constituting a mass far greater than that of the known universe²⁶, evolution techniques must target specially selected segments of the chosen starting scaffold. That is, the experimental strategy for efficient *in vitro* evolution should combine rational design and selection in the experimental strategy for *in vitro* evolution.

We used the elements of a pre-existing binding site for the phosphate and anthranilate structural motifs to simplify the design. CdRP is the product of PRAI and so the binding site of PRAI must also bind CdRP. IGPS binds CdRP, therefore, we reasoned that it has the potential to bind PRA. The next rational component of the design was derived from the detailed comparative analysis of structural and biochemical data on IGPS and PRAI^{12,13,15,17–21}. Using this information, we superimposed the structures of IGPS and PRAI using the program SETOR (Fig. 2). There are three regions in the scaffold of IGPS that are strikingly different from those in PRAI. The IGPS active site has an N-terminal extension of 49 residues, including an N-terminal α 0 helix of five turns; the β 1 α 1 connecting sequence is a long loop of 15 residues; and the β 6 α 6 connection of 11 residues is in a completely different orientation from the equivalent segment in PRAI. All these sequences are located at the C-terminal side of the strands of the β -barrel. PRAI, however, has an entirely different active-site loop system: it has only a four-residue N-terminal extension; the β 1 α 1 connection is just four residues; β 6 α 6 (11 residues) is rich in glycine and has a highly conserved consensus sequence GXGGXGQ. The similarities are the β 2 α 2 loop, which is involved in binding the anthranilic acid moiety of the substrates PRA and CdRP, and the β 8 α 8 and the β 7 α 7 loop, which comprise the phosphate binding site (Fig. 2). But there is an additional α 8' helix in IGPS that alters the orientation of the bound phosphate. From this comparison, we selected the active-site loops in each protein as our targets. As the

loops (β 2 α 2, β 7 α 7 and β 8 α 8) are similarly arranged in the two enzymes, our targets were solely the extra N-terminal end (helix α 0 and two bends), the β 1 α 1 loops and the β 6 α 6 loops. Our approach mimics natural evolution, creating genetic diversity by using deletion, insertion, recombination and selection^{5–7,32}. The strategy to confer PRAI activity on the IGPS scaffold is shown in Fig. 3.

The first step in the design was the deletion of 48 amino-acid residues from the N-terminal end of IGPS to create the deletion mutant IGPS49. This mutant was unstable, had a tendency to aggregate³¹ and was catalytically inactive with respect to both IGPS and PRAI. The gene encoding IGPS49 was expressed in *Escherichia coli*, but the protein formed inclusion bodies that could not be renatured by conventional procedures. Refolding chromatography with immobilized minichaperones was developed to renature the protein quantitatively³³. It had a circular dichroism spectrum characteristic of a native α/β -barrel protein and bound ³H-rCdRP, a specific inhibitor of IGPS, with a stoichiometry of one mole of inhibitor per mole of IGPS49 (data not shown).

The next step was to make a complex library composed of three different component libraries that involved shortening the β 1 α 1 loop by deleting 15 and inserting 4–7 residues. We decided to introduce the diversity in length to allow flexibility both at the active site and in the subsequent recombination steps, described below. All the inserts have the common motif GK, which favours flexibility and provides the conserved lysine of the PRAI family. This family has a conserved sequence pattern KXC (Lys-X-Cys); IGPS has CKK in the same position. We decided to exchange the first K for G (a wild-card residue) to mimic this feature. The library IGPS49L1 has GKXXG; IGPS49L1RGD has GKXRGD; and IGPS49L1SV has a mixture of GKXX, GKXXX, GKXXXX or GKXXXXX. We used PCR methodologies including overlap-extension polymerase chain reaction (PCR), inverse PCR and random-primer PCR. The libraries were analysed by PCR screening, by restriction analysis and by sequencing. Members of each library were picked at random and expressed in *E. coli*. The proteins appeared in the soluble fraction

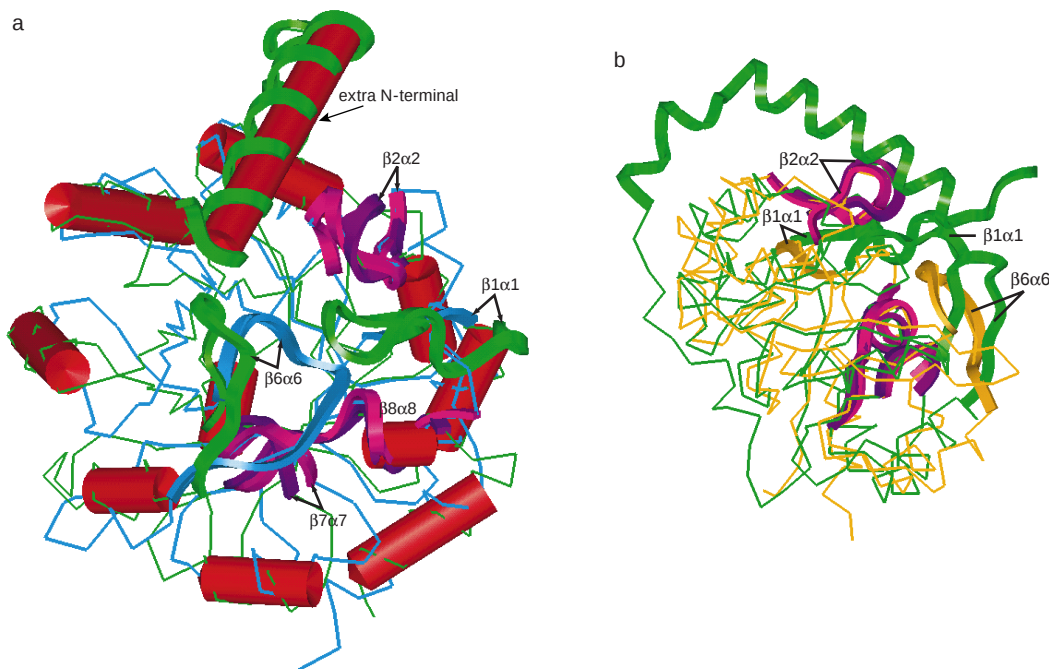


Figure 2 Superimposed C α traces of IGPS (residues 1–255, green line) and PRAI (residues 255–452, blue line). **A**, Top view. The α -helices of IGPS are in red. The active-site loop regions (ribbon representation) in both proteins are shown: loops β 1 α 1 and β 6 α 6 (IGPS, green; PRAI, blue) and extra N-terminal: helix α 0 of IGPS (green ribbon). Phosphate (β 7 α 7, β 8 α 8) and anthranilate (β 2 α 2) binding sites are depicted in violet

(IGPS) or in magenta (PRAI). **B**, Side view of the superimposed C α traces of IGPS (residues 1–255, green) and PRAI (residues 255–452, blue). The different components of the lid in IGPS completely cover the active site; in contrast, the active site in PRAI is partially uncovered.

but were prone to aggregation above a concentration of 0.5 mg ml^{-1} . We denatured one of the protein samples in 8 M urea, and renatured it using refolding chromatography³³. The refolded protein was soluble and able to bind ^3H -rCdRP, but it lacked catalytic activity.

The next set of modifications that we made, of the $\beta 6\alpha 6$ loop, was on the combined mixture of libraries. We introduced an aspartic residue at position 184 (a general base in the active site)^{13,27} and the PRAI consensus sequence GXGGXGQ²⁷ in an attempt to improve the active-site loop system. A new library called IGPS49L1L6 was constructed using the IGPS49L1, IGPS49L1RGD and IGPS49L1SV libraries as templates. One of the new library members chosen at random was expressed in *E. coli*; the corresponding protein was found to be soluble, and it had a circular dichroism spectrum characteristic of a typical α/β -barrel protein. Furthermore, it was able to bind ^3H -rCdRP but lacked the activity of either PRAI or IGPS.

Directed evolution

Having introduced the basic design of the PRAI loop system into IGPS, we initiated the first cycle of selection. We designed an *in vivo* selection strategy for PRAI activity that was based on complementation of *E. coli* strain JA300 (a PRAI-deficient strain that does not grow in the absence of tryptophan (Trp)). In *E. coli*, PRAI and IGPS are part of the same polypeptide chain (relative molecular mass $45,000$ (M_r 45K)) specified by the *trpC* gene; however, *E. coli* strain

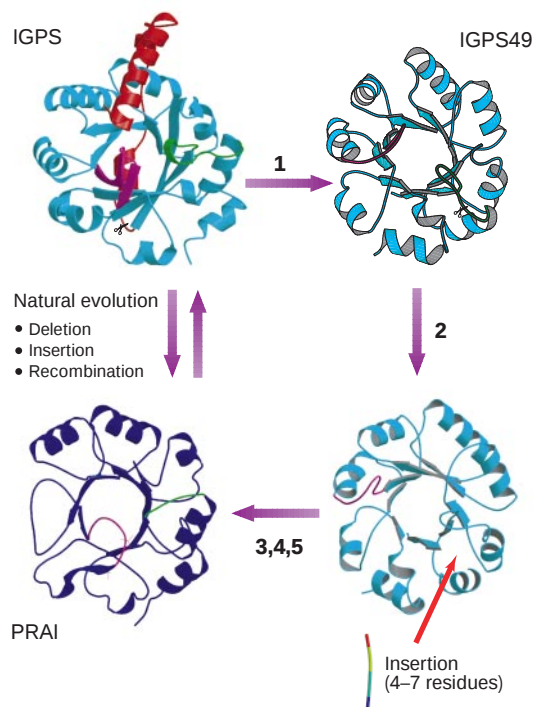


Figure 3 Ribbons representation of the experimental strategy for evolving a new function in the IGPS scaffold. Top left, initial IGPS scaffold. Extra N-terminal residues are red, loop $\beta 1\alpha 1$ is green and loop $\beta 6\alpha 6$ is magenta. Bottom left, PRAI scaffold. Loop $\beta 1\alpha 1$ is green and loop $\beta 6\alpha 6$ is magenta. **1**, Deletion of 48 amino acids (red structure) from the N terminus of IGPS, yielding IGPS49. **2**, Deletion of 15 amino acids corresponding to loop $\beta 1\alpha 1$ and replacement with 4–7 residues, yielding the libraries IGPS49L1 (GXGXG), IGPS49L1RGD (GXKRGD) and IGPS49L1SV (length varies). **3**, Introduction of an aspartic residue in position 184 and replacement of loop $\beta 6\alpha 6$ by the PRAI consensus sequence GXGGXGQ. **4**, *In vivo* selection experiments by complementation of strain JA300 (a PRAI-deficient strain that does not grow in the absence of tryptophan) and plating colonies on minimal medium with ampicillin, streptomycin, IGPS and a range of tryptophan concentrations. **5**, *In vitro* recombination (DNA shuffling and STEP PCR) to fit barrel shape and improve its function, yielding ivePRAI.

JA300 carries the W3110(*trpC1117*) allele and so lacks isomerase activity, but retains normal levels of synthase activity^{34–36}. Complementation should, therefore, indicate that the specific clone contains a plasmid expressing an IGPS variant with PRAI activity. JA300, itself, showed no ability to grow in the absence of tryptophan (Fig. 4A). The initial parental clones (IGPS49, IGPS49L1, IGPS49L1RGD and IGPS49LSV) failed to grow in the absence of tryptophan (Fig. 4B). The DNA library IGPS49L1L6 was used to transform the JA300 strain. About 3×10^4 *E. coli* transformants

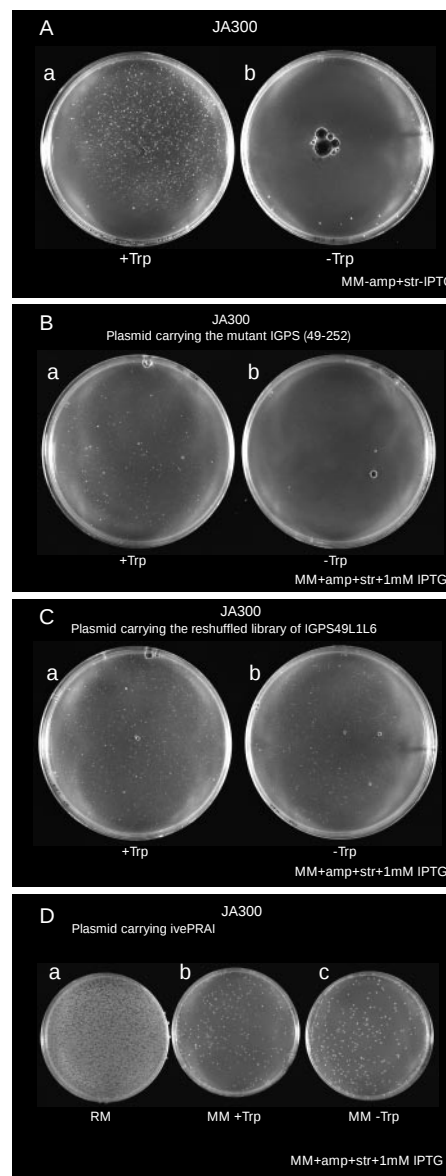


Figure 4 Complementation-based *in vivo* selection of active PRAI-containing clones using *E. coli* JA300 (a PRAI-deficient strain that does not grow in the absence of tryptophan). **A**, JA300 cells were plated on M9 medium containing streptomycin ($25 \mu\text{g ml}^{-1}$), leucine ($20 \mu\text{g ml}^{-1}$) and threonine ($25 \mu\text{g ml}^{-1}$) in the presence (**a**) or absence (**b**) of tryptophan ($25 \mu\text{g ml}^{-1}$). **B**, JA300 cells transformed with plasmid carrying IGPS49 were plated in the same medium plus ampicillin and IPTG in the presence (**a**) or absence (**b**) of tryptophan. **C**, JA300 cells transformed with the plasmid carrying the reshuffled library IGPS49L1L6 from the first cycle of reshuffling were plated in the presence (**a**) or absence (**b**) of tryptophan ($25 \mu\text{g ml}^{-1}$). **D**, JA300 cells transformed with plasmid carrying the reshuffled library IGPS49L1L6 from the second cycle were plated (**a**) in rich medium (ZTY) plus ampicillin and IPTG or in minimal medium in the presence (**b**) or absence (**c**) of tryptophan ($25 \mu\text{g ml}^{-1}$).

expressing the resultant library were then plated on minimal medium containing a range of tryptophan concentrations (0–25 $\mu\text{g ml}^{-1}$). The colonies (~ 500) growing at low tryptophan concentrations were selected.

The second step of our procedure was non-rational design (Fig. 3). This is the 'fine tuning' to improve the fit and the new function of the scaffold by *in vitro* evolution methods, including *in vitro* sexual recombination and selection cycles. A first round of DNA shuffling was carried out with the pool of genes from the selected clones using published methods^{16,17,22}. Plating about 4×10^5 bacteria on a wide range of tryptophan concentrations yielded 80 colonies. These were able to grow at very low concentration of tryptophan ($< 1 \mu\text{g ml}^{-1}$) and a single clone was capable of growing in the absence of any exogenous tryptophan. Restriction-fragment length polymorphism analysis of 30 clones chosen at random revealed a minimum of eight different patterns. A second round of recombination was carried out by DNA shuffling¹⁷ and staggered extension procedure (StEP)²¹, using the pool of 80 colonies selected from the first round and synthetic DNA fragments encoding the protein segments corresponding to loop $\beta 4\alpha 4$ from diverse species of PRAI. ($\beta 4\alpha 4$ was one of the loops that we had not targeted, and this PRAI segment was not found to be incorporated into the selected final clones.) The *in vivo* selection found 360 colonies that were capable of growing in the absence of any exogenous tryptophan (Fig. 4C). We carried out several controls to show that the ability to grow in absence of tryptophan is a consequence of the introduction of the reshuffled library (IGPS49L1L6-2 cycle) containing the ivePRAI (*in vitro* evolved PRAI) genes. As a first control, we cured the JA300 strain that was previously transformed with the plasmids carrying the library by growing the bacteria in the absence of ampicillin. The cured cells were unable to grow in ampicillin-containing medium and simultaneously lost the ability

to grow in the absence of tryptophan. Furthermore, the plasmid carrying the ivePRAI gene was used to transform fresh JA300 cells, before plating on minimal medium with added ampicillin, streptomycin and isopropyl- β -D-thiogalactoside (IPTG), but in the absence of tryptophan. These transformed cells could grow for 18 h in the absence of tryptophan, all the clones were ampicillin resistant and were *Trp*⁺ (Fig. 4D) (see additional controls in Methods). On the basis of these controls, it seems reasonable, therefore, to infer that the PRAI activity complementing the auxotrophy in JA300 cells originates from the cloned IGPS variant genes and is not the product of any reversion event.

In vitro evolved PRAI

We sequenced genes encoding the ivePRAI proteins from 30 clones, and found only 8 different sequences. The largest colony from a plate of minimal medium without tryptophan was selected for further biochemical characterization. The gene encoding the ivePRAI was expressed and the protein purified. The new protein was soluble, and the circular dichroism spectra and the activity assay confirmed that it was properly folded.

The ivePRAI has PRAI activity and does not have IGPS activity *in vitro*. ivePRAI has a specificity constant (k_{cat}/K_m) of $4.8 \times 10^7 \text{ s}^{-1} \text{ M}^{-1}$ (Table 1), which is sixfold that of either the natural enzyme (*E. coli* wild-type bi-enzyme) or the isolated PRAI domain (Table 1). This improved activity results primarily from a 15-fold enhanced affinity of the evolved protein for PRA (Table 1).

The structure of ivePRAI resembles IGPS and differs significantly from that of PRAI. ivePRAI has 28% sequence identity to PRAI, and 90% to IGPS (Fig. 5). Notably, the binding site for the phosphate ion in the IGPS scaffold of ivePRAI is at the N-terminal turn of the additional α -helix $\alpha 8'$ that is located in the loop between strand $\beta 8$ and helix $\alpha 8$. The wild-type PRAI has a shorter and less-

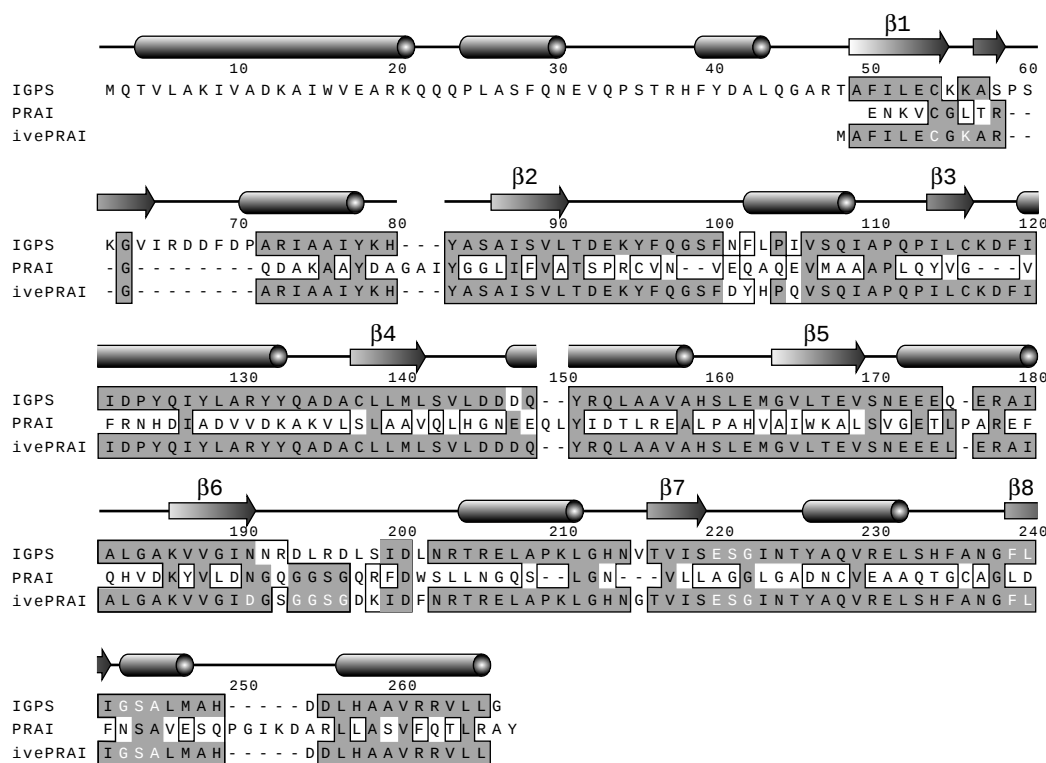


Figure 5 Sequence alignment of *in vitro* evolved PRAI (ivePRAI), wild-type PRAI and wild-type IGPS. IGPS (residues 71–254) with ivePRAI (residues 1–196); identities 167/184 (90%); similarities: 171/184 (92%). PRAI (residues 375–396) with ivePRAI (residues 119–136); identities 8/18 (44%); similarities 12/18 (66%). Results are from the program BLAST⁴². Identities are outlined and shaded; similarities are outlined. The catalytic

residues are shown in white. IGPS secondary structure is indicated. Note that the mutation of N to D at position 184 of IGPS appears at position 190 in the figure because there are six blank insertions in its sequence to align with PRAI. Note also that this catalytic aspartate is frameshifted in ivePRAI with respect to PRAI, showing that the active sites are different.

Table 1 Kinetic properties of wild-type IGPS, PRAI and ivePRAI

Enzyme	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($s^{-1}M^{-1}$)	K_i^* (μM)
IGPS reaction				
Wild-type bi-enzyme†	3.8	0.44	8.6×10^6	0.19
IGPS (1–252)†	4.3	13	3.3×10^5	nd
IGPS (1–254)‡	4	0.5	8×10^6	0.33
ivePRAI§	0	0	0	0.39
PRAI reaction				
Wild-type bi-enzyme	40	4.9	8.2×10^6	6.5
PRAI (256–452)†	32	4.7	6.8×10^6	6.8
IGPS (1–254)†	0	0	0	n.d.
ivePRAI§	15.3	0.32	4.8×10^7	0.39

* K_i of competitive inhibitor 3H -rCdRP.

† See ref. 14.

‡ IGPS–Flag Tag (residues 1–259).

§ IGPS scaffold–Flag Tag (residues 49–Δ10–254).

ordered additional α -helix ($\alpha 8'$) than has IGPS; accordingly, the bound phosphate moiety has a different orientation²⁷. Further, the site for binding the anthranilate moiety of PRA in ivePRAI is also inherited from IGPS and is quite different from that of PRAI (Fig. 2). Despite these differences, the catalytic constants of ivePRAI and PRAI are similar (Table 1).

We have thus shown experimentally that two classes of α/β -barrels can be interconverted by altering the loop regions. Our results demonstrate the divergent evolution of two enzymes from the pathway for the biosynthesis of tryptophan, which may mimic natural divergent evolution³⁷. For *in vitro* design purposes, we successfully evolved a new function in the scaffold of an α/β -barrel protein using the combined approach of rational design, *in vitro* mutation, recombination and *in vivo* selection. Perhaps the directed evolution methods used in this work will allow progress to be made in protein engineering through routes not open to Nature, thus potentially allowing access to more efficient catalytic solutions, as in the example presented here (Table 1). It has long been speculated that α/β -barrel proteins may be a useful scaffold for evolving binding and catalysis *in vitro*³⁸. Perhaps our strategy of targeting loop replacements may be of wider application. □

Methods

Reagents

Restriction enzymes and T4 DNA ligase were obtained from BioLabs. *Taq* polymerase and Wizard DNA preparation kits were obtained from Promega. Ultrapure dNTPs were obtained from Boehringer Mannheim. DNase I and other reagents were obtained from Sigma.

Chemical syntheses

rCdRP and 3H -rCdRP were prepared as described³⁹. The specific activity of the 3H -rCdRP was 95.36 kBq μmol^{-1} .

Preparation of DNA

The gene encoding IGPS (residues 1–259) was amplified from *E. coli* strain BL21 genomic DNA by PCR (94 °C, 1 min; 37 °C, 1 min; 72 °C, 1 min; 25 cycles) using the primers 'IGPSFULL' and 'IGPSFLAGREV'. The PCR product was digested with *NcoI* and *BspHI* and the 820-basepair (bp) fragment was cloned into the *NcoI* site of pNS3785 (ref. 40) to create pJB122. pJB122 thus encodes a polypeptide chain comprising residues 1–259 of IGPS fused directly to the Flag-tag GSDYKDDDDK at the C terminus of IGPS. The gene encoding IGPS49 (residues 49–259) was amplified by PCR from pJB122 using the primers 'IGPS49FSP1' and 'JB122SEQ'; and was then digested with *FspI* and *BamHI*. pJB124 was created by ligation of the 630-bp PCR fragment with a 4,700-bp fragment generated from pJB122 by digestion with *NcoI*, blunt-ending with Klenow polymerase, and further digestion with *BamHI*. The gene encoding IGPS49 was used as a template for further modifications and re-cloned in the same vector described above, and a set of different plasmids (pMA) carrying all the libraries were created.

Oligonucleotides

The following oligonucleotides were used: IGPSFULL, 5'-CATGACCTTGCGGCC-CAGCCGCCATGGCGCAACCGTTTATGCGAAATCGTCGC-3'; IGPSFLAGREV, 5'-ATCGTCATAATCATGAACCTACTGTGTCATCGTCCTTGTAGTCGGA-TCCTACTTTATCTCACCAGCAACACCCGGCGCACGG-3'; IGPS49L1,

5'-NNSNNSNNSGGTGCACGCATTGCCGCCATTATAAACATTACGC-3'; IGPS49Lr, 5'-ACCGCACTCCAGAATAAATGCCCTTCC-3'; IGPS49FSP1, 5'-CATGACCTTGTGCGCATTTATTCTGGAGTGC-3'; JB122SEQ, 5'-CCCTGCGGCTGGTAATGG-3'; IGPS49L1L6r, 5'-CCCACCSNNGCCGTTGATGCCAACGACCTTTGCCCC-3'; IGPS (ApaI), 5'-CGCCGTGCGTGCACCTGTAGCGC-3'; L1 (6aa), 5'-GGAAGG-CATTATTCTGGAGTGGCGTNNNSNNSNNSGGTGCACGCATTGCCGCC-3'; L1APAL1, 5'-TTTATTCTGGAGTGGCGTCTANNNSNNSNNSGGTGCA-CGCATTGCCGCC-3'; L1APALre, 5'-GGCGGCAATGCGTGCACCSNN-SNNSNNTAGACCGCACTCCAGAATAAA-3'; L6, 5'-GCAAAGTTCGTTGGCATCAACGGCNSGGTGGGNSNNSNNSATTTGATCT-CAACCGTACC-3'; L6rev, 5'-GGTACGGTTGAGATCAATSNNSNNSNACCSNNCC-CACCSNNGCCGTGATGCCAACGACCTTTCG-3'.

DNA shuffling

The shuffling of the pool of genes from the first cycle of selection was carried out¹⁶ using 60–80-bp fragments generated by DNase I (Sigma) and reassembled by PCR without added primers. A PCR program of 95 °C, 1 min, 40 cycles (94 °C, 30 s; 55 °C, 30 s; 72 °C, 1 min + 5 s; per cycle) was used. After 40-fold dilution of the minus primer product into a PCR mix with 1 μM of each primer and 20 additional cycles of PCR (94 °C, 30 s; 55 °C, 30 s; 72 °C, 2 min), a single product of 650 bp was obtained. The shuffled material was cloned back into the vector described above and used to transform the PRAI-deficient *E. coli* strain JA300 (refs 34, 41).

The second cycle of shuffling was carried out on the pool of chimera selected in the first round and synthetic DNA fragments encoding for the protein segments corresponding to loops $\beta 1\alpha$, $\beta 6\alpha$, $\beta 4\alpha$ from diverse species of PRAI.

Staggered extension process (StEP)

The StEP conditions were as described²¹. A PCR program of 92 cycles (94 °C, 30 s; 55 °C, 4 s) was used. At this step, we removed the parent DNA (purified from a *dam*⁺ strain) using *DpnI*. A second PCR was performed by adding primers to amplify the full-length product (95 °C, 2 min; 25 cycles (94 °C, 30 s; 55 °C, 1 min; 72 °C, 5 min) 72 °C, 30 min).

Selection

JA300 cells were plated on minimal medium (M9) with ampicillin (50 $\mu g ml^{-1}$) and streptomycin (20 $\mu g ml^{-1}$) plus 0.7 mM IPTG, containing a range of tryptophan concentrations, and incubated at 37 °C for 24–36 h. About 500 colonies from the plates with the lower tryptophan concentrations were pooled and cultured either in liquid medium 2 $\times$ TY + ampicillin + streptomycin or M9 + ampicillin + streptomycin + 0.7 mM IPTG with the similar level of tryptophan. Plasmid DNA was prepared from this liquid culture.

Additional control experiments

We prepared plasmid DNA from the pool of clones selected after the second round of recombination and used this DNA to transform fresh JA300 cells, before plating on minimal medium with added ampicillin, streptomycin and IPTG but in the absence of tryptophan. These transformed cells were able to grow in the absence of tryptophan for 18 h. Additionally, we purified the plasmid DNA from these cells, excised the insert by restriction digestion and re-cloned it into a fresh vector. After transforming this vector into fresh JA300 cells, we obtained positive clones in the absence of tryptophan, showing that the activity is insert dependent. The same result was obtained when the DNA was amplified by PCR, re-cloned and introduced into fresh JA300 cells.

Refolding chromatography

Protein renaturation was done as described³³.

Protein purification

After refolding experiments, the proteins (IGPS49, IGPS49L1 and IGPS49L1L6) were purified as described³⁹.

PRAI activity assay

All the kinetic and binding experiments were done as described^{12,15}.

Sequence alignment

The amino-acid sequences of ivePRAI, IGPS and PRAI were aligned using a sequence-similarity search of SCOP sequences based on the BLAST algorithm⁴². We show the sequence alignment based on ClustalW algorithm (Matrix Blosom 30) in Fig. 5.

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A comprehensive analysis of protein–protein interactions in *Saccharomyces cerevisiae*

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Two large-scale yeast two-hybrid screens were undertaken to identify protein–protein interactions between full-length open reading frames predicted from the *Saccharomyces cerevisiae* genome sequence. In one approach, we constructed a protein array of about 6,000 yeast transformants, with each transformant expressing one of the open reading frames as a fusion to an activation domain. This array was screened by a simple and automated procedure for 192 yeast proteins, with positive responses identified by their positions in the array. In a second approach, we pooled cells expressing one of about 6,000 activation domain fusions to generate a library. We used a high-throughput screening procedure to screen nearly all of the 6,000 predicted yeast proteins, expressed as Gal4 DNA-binding domain fusion proteins, against the library, and characterized positives by sequence analysis. These approaches resulted in the detection of 957 putative interactions involving 1,004 *S. cerevisiae* proteins. These data reveal interactions that place functionally unclassified proteins in a biological context, interactions between proteins involved in the same biological function, and interactions that link biological functions together into larger cellular processes. The results of these screens are shown here.

With the completion of the genome sequence of the budding yeast *Saccharomyces cerevisiae* in April 1996 (ref. 1), a eukaryotic organism could be analysed on a genomic scale for the first time. The challenge became one of understanding the roles of the approximately 6,000 gene products and how they interact to create a eukaryotic organism. However, one-third of the predicted yeast open reading frames (ORFs) are still classified as proteins of unknown function². Genomic technologies have been developed that focus predominantly on the use of DNA array approaches to measure, for example, the expression of large sets of genes. As comparable strategies for protein analysis are not available, we initiated two types of systematic study to produce additional information that can place yeast ORFs within a biological context, with the goal of understanding their functional roles. We chose to use the yeast two-hybrid system because it can identify pairs of proteins that physically associate with one another³ and because two-hybrid screens are simple, sensitive and amenable to high-throughput methods. The first genomic analysis using the two-hybrid system centred on the T7 bacteriophage⁴. Large complexes in *S. cerevisiae* have also been analysed using this method of detecting protein–protein interactions^{5,6}. Here we present the results of two complementary strategies using the two-hybrid screen to identify protein–protein interactions among the predicted ORFs of *S. cerevisiae*. A bioinformatics platform for the analysis of this data set is publicly available at <http://portal.curagen.com>.

A protein array of activation-domain hybrids

To examine protein activity in a format that allows the assay of every predicted ORF, we constructed an array of hybrid proteins. At least two general types of protein array may be envisioned: those composed of living transformants, as described here, with each protein expressed in a form that allows expedient assay of the host

cells; and those composed solely of the purified proteins⁷. The two-hybrid array used here is a set of yeast colonies derived from about 6,000 individual transformants. The transformation event inserts one of the yeast ORFs into a Gal4 transcription-activation domain vector to create a hybrid protein. To enable rapid, large-scale transformation, we generated those ORFs as a set of polymerase chain reaction (PCR) products with 70 base-pair sequences at their 5' and 3' ends that precisely matched sequences in the activation domain vector pOAD⁸. These sequences allowed highly efficient recombination between the ORFs and the linearized vector. After transformation of a yeast two-hybrid reporter strain⁹, we pooled two colonies from each transformation plate to constitute a single array element (Fig. 1a), with the entire array contained on sixteen microassay plates of 384 colonies each.

A set of 192 DNA-binding domain hybrids was similarly generated by transformation into a strain of opposite mating type of PCR products with a Gal4 DNA-binding domain vector. To screen for protein interactions, we mated a transformant containing one of the DNA-binding domain hybrids to all of the transformants of the array, selecting diploids using markers carried on the two-hybrid plasmids. The diploids were then transferred to selective plates deficient in histidine, and colonies positive for the two-hybrid reporter *HIS3* gene were identified by their positions in the array (Fig. 1b). For each of the 192 screens, we typically obtained on the order of 1–30 positives. However, only around 20% of these positives were reproduced in a second screen of the array (Table 1a). Although the exact causes of this variability are not known, they appear to include an infrequent and protein-specific rearrangement of the DNA-binding domain plasmid to generate proteins that activate transcription on their own. As a consequence of this variability, we scored as putative interacting partners only those proteins that were identified in two independent screens, even though this criterion also resulted in the omission of some known interactions found only once. Overall, 87 of the 192 DNA-binding domain hybrids screened were identified in a putative

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protein–protein interaction, resulting in 281 interacting protein pairs (Table 1a).

High-throughput screens of an activation-domain library

As an alternative method of genomic two-hybrid analysis, we developed high-throughput screens based on a library made by pooling transformants containing the roughly 6,000 potential ORFs fused to the Gal4 activation domain. The hybrid proteins were derived similarly to those in the two-hybrid array, with each of the potential ORFs cloned separately into a Gal4 DNA-binding domain vector in addition to the Gal4 activation domain vector. The same PCR products and recipient plasmids were used to generate two collections of transformants, each consisting of 64 barcoded 96-well plates. Of the 6,144 ORFs, 5,345 (87%) were successfully cloned into both plasmids (Table 1b). Transformants from the Gal4 activation domain collection were then pooled to form an activation-domain library. To screen for protein interactions, we mated each DNA-binding domain hybrid transformant in duplicate to the activation domain library. Mating mixes were transferred to selective plates to select diploid cells that expressed interacting pairs and activated both reporter genes (*URA3* and *lacZ*). Experiments were conducted in 96-well assay plates using semi-automation and computerized sample tracking to perform such large-scale transformation and mating reactions quickly.

Overall, 817 yeast ORFs (15% of the successfully cloned yeast ORFs) were identified in a putative protein–protein interaction by this approach, resulting in 692 interacting protein pairs (Table 1b). To assess the coverage of the screens, we classified the interactions according to their frequency. Sixty-eight per cent of the putative interactions were identified in independent experiments (41%) or multiple times in a single experiment (27%). The remaining 32% of the interactions were identified only once during the screening process. This moderate coverage reflects the depth of the experiment determined by the number of colonies per mating submitted for sequence analysis. To complete this study in a timely and cost-effective manner, we selected only 12 colonies from each mating. This number was chosen to validate this genome-wide high-throughput approach as well as to generate significant data of scientific interest.

Comparison of the approaches

The array screens and the library screens gave different data sets (Table 2). Forty-five per cent of the 192 proteins used in the array screens yielded interactions, compared to 8% of the 5,345 potential ORFs in the library screens. Some of the difference in the number of

proteins that resulted in positives with each approach is attributable to the nonrandom choice of proteins for the array screens (some categories of proteins, such as membrane proteins and metabolic enzymes, are less likely to yield interacting partners, whereas signalling proteins are more likely to). However, for proteins that identified at least one binding partner, the array screens gave an average of 3.3 positives per protein, whereas the library screens gave an average of 1.8. In addition, the 12 positive DNA-binding domain hybrids common to both screens yielded 48 putative interactions in the array screens and 14 in the library screens. Thus, although the library approach permits a much higher throughput, the array screens generate more candidate interactors. The higher yield of candidates in the array screens can be partially attributed to the stringency of the selection procedures; the His selection for 14 days in the array screens was less stringent than the Ura selection for 4 days in the library screens. More significantly, the pooling of activation-domain transformants may select against interactions that involve cells with reduced growth rate or mating ability, and sequence analysis of twelve positives may identify only strongly interacting pairs. In this regard, the array may facilitate the detection of interactions that result in very low reporter gene activation, in that a single positive array element on the two-hybrid selection plate may be composed of many small, slow-growing colonies. However, the

Table 1 Summary of experimental results

(a) Protein array	
Description	Total
Yeast ORFs screened	192
Yeast ORF yielding reproducible interactions	87
Total discrete interacting protein pairs	281
(reproduced in a second screen)	
(b) High-throughput screens	
Description	Total
Yeast ORF PCR products	6,144
Yeast ORFs cloned*	5,345
ORFs pooled to generate the activation domain library	5,341†
ORF activating transcription on their own	680
Yeast ORFs identified to have interactions‡	817
Total discrete interacting protein pairs	692
Interactions identified in independent experiments§	286
Interactions identified multiple times in a single experiment	186
Interactions identified only once	220

* Number of PCR products giving transformants in both plasmids (pOBD2 and pOAD).

† One yeast ORF activation domain construct was excluded from the pool owing to self-activation in a test screen (YJR009C—glyceraldehyde-3-phosphate dehydrogenase) and three yeast ORF activation domain constructs were excluded because they encoded proteins that could affect the selection process (YPL248C—Gal4, YML051W—Gal80 and YEL021W—Ura3).

‡ The total number of yeast ORFs found as interacting binding domain clones and/or interacting activation domain clones in the screens.

§ All screening experiments were performed in duplicate.

|| Interactions that were identified several times in only one mating.

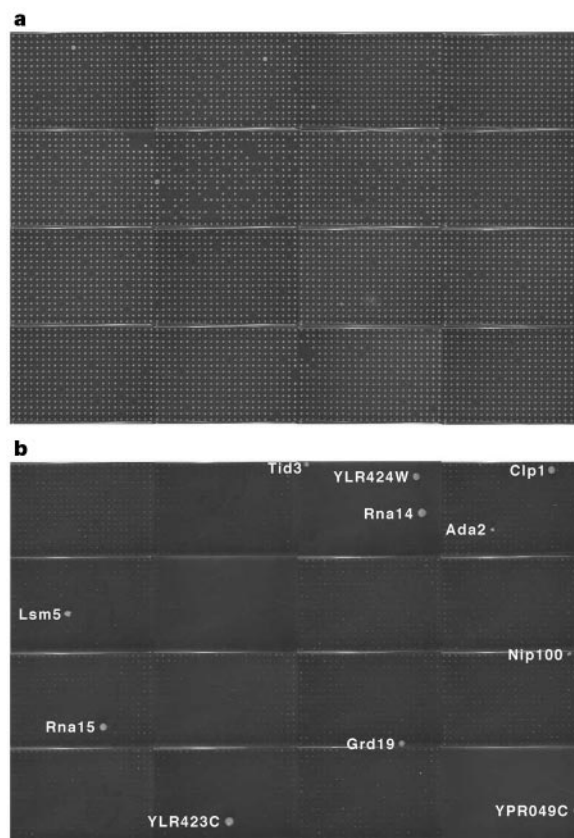


Figure 1 The two-hybrid assay carried out by screening a protein array. **a**, The array of ~6,000 haploid yeast transformants plated on medium lacking leucine, which allows growth of all transformants. Each transformant expresses one of the yeast ORFs expressed as a fusion to the Gal4 activation domain. **b**, Two-hybrid positives from a screen of the array with a Gal4 DNA-binding domain fusion of the Pcf11 protein, a component of the pre-mRNA cleavage and polyadenylation factor IA, which also consists of four other polypeptides³⁶. Diploid colonies are shown after two weeks of growth on medium lacking tryptophan, leucine and histidine and supplemented with 3 mM 3-amino-1,2,4-triazole, thus allowing growth only of cells that express the *HIS3* two-hybrid reporter gene. Three other components of factor IA, Rna14, Rna15 and Clp1, were identified as Pcf11 interactors. Positives that do not appear in Table 2 were either not reproducible or are false positives that occurred in many screens.

array screens are much more labour- and material-intensive, and require several hours of robot time per screen, thus severely limiting the number of screens that can be performed.

We were interested in examining the putative protein–protein interactions identified in these screens in reference to their functional roles according to the yeast protein database (YPD) classification¹⁰. Representative proteins from 41 of the 43 YPD categories were identified in the screens (Table 3). Of the 1,004 active proteins, 412 fell into the ‘unclassified’ category. These 412 proteins yielded 509 distinct interactions, of which 164 (32%) were between proteins with no functional classification. This observation indicates that there may be a significant number of as yet undiscovered pathways and/or complexes that can be identified using systematic approaches. Results from both approaches were also compared with a compilation of literature-cited interactions (Table 2). From the 957 putative interactions identified by both

approaches, at least 109 have been previously reported by others using two-hybrid, co-immunoprecipitation, copurification or affinity column techniques^{2,10}. That only a subset of previously described interactions has been detected in our work can be attributed to specific features of the screens: the exclusive use of full-length proteins as both DNA-binding and activation-domain fusions and the versions of the two-hybrid system used, which include Gal4 as the DNA-binding domain fusion protein and centromeric plasmids. Each of these components can affect the sensitivity of the assay¹¹. Additionally, sequence analysis of 200 ORF constructs used in constructing the array indicates that ~15% of the recombinant plasmids may lack insert, another ~3% may contain frameshifting errors, and about 5% of all colonies failed to grow. Thus, we estimate that the array contains ~85–90% of the yeast ORFs, given that each element is composed of two individual transformants.

Results of the systematic two-hybrid screens

To examine the large number of potential interactions, we developed a new bioinformatics platform which, along with the complete set of data generated by these screens, is publicly available at <http://portal.curagen.com>. The software allows users to search for information on putative protein interactions identified in the screens or reported in the literature, to perform sequence analyses and view the results, to extend interactions to construct pathways and to view the homologues of the yeast genes in a number of species using the three-dimensional homologue viewer (see Fig. 2).

First, by using systematic approaches to analyse the yeast genome, we could identify interactions that place functionally unclassified proteins into a biological context. For example, two proteins of unknown function, YGR010W and YLR328W (77% identical), were observed to interact with each other. Additionally, both proteins bind to ornithine aminotransferase (Car2), indicating that they may be involved in arginine metabolism. Human ornithine aminotransferase (OATase) can complement an ornithine aminotransferase-deficient strain of *S. cerevisiae*, and mutations in human OATase cause gyrate atrophy of the choroid and retina¹².

Data from this study provide evidence of links between two proteins involved in autophagy, Apg13 and Apg1, and proteins of the Cvt (cytoplasm-to-vacuole targeting) pathway, Lap4, Vma22 and Vma6. Autophagy is a degradation pathway used under conditions of nutrient stress to nonselectively recycle cytoplasmic proteins and organelles to their constituent components, whereas the Cvt pathway is a biosynthetic pathway that transports the vacuolar enzyme aminopeptidase I (API, encoded by *LAP4*) specifically to the vacuole¹³. Several mutations in the Cvt pathway (*cvt*) and autophagocytosis (*aut* and *apg*) are allelic, indicating that both pathways may utilize some of the same molecular components¹⁴. Our study implicates a number of ORFs encoding proteins of unknown functions as potential components of autophagy (Fig. 3a). As several of the genes altered in *apg*, *aut* and *cvt* mutants have not yet been cloned, ORFs found in these interactions could be examined to determine whether they encode any of these genes. This study has also shown Lap4 to be a self-interactor, corroborating previous evidence that Lap4 assembles into a dodecamer¹⁵, and the interaction between Apg1 and Apg13 lends support to previous genetic evidence indicating that *APG1* may be a high-copy suppressor of *apg13* (ref. 16).

Second, genome-wide two-hybrid approaches offer insight into novel interactions between proteins involved in the same biological function. For example, we screened four proteins (Lsm2, Lsm4, Lsm8 and Prp11) that are known or suspected to be involved in RNA splicing and that had been previously analysed or identified in another systematic set of two-hybrid screens⁵ using a random library of activation-domain hybrids. Three of these contain the Sm1 and Sm2 motifs found in a core set of proteins associated with small nuclear RNAs (snRNAs) involved in splicing^{17,18}; the yeast Sm proteins are homologous to the eight common Sm proteins identified

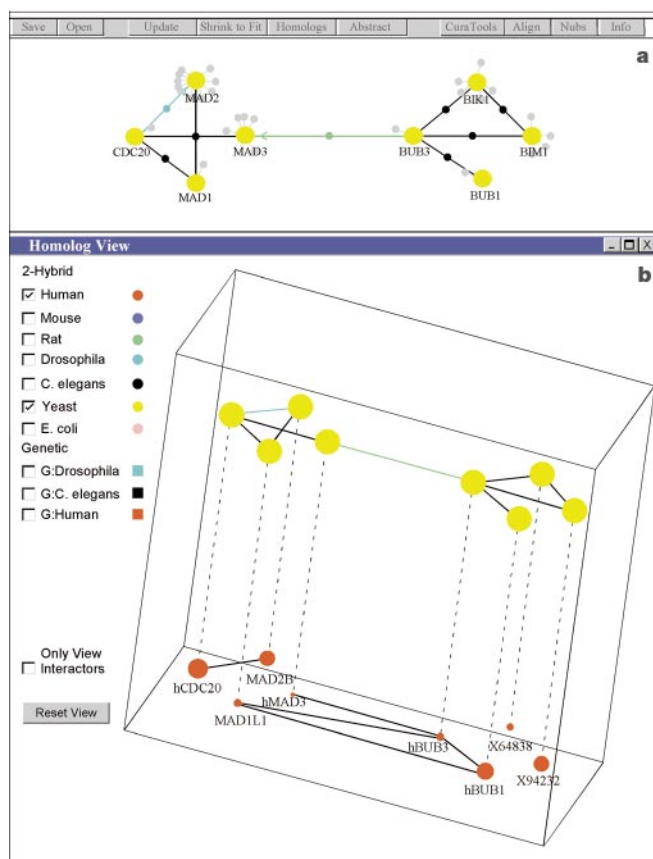


Figure 2 Data analysis software. **a**, The putative interaction identified between Mad3 and Bub3 which connects the spindle checkpoint complex³⁷ and the microtubule checkpoint complex³⁸. Yeast proteins are shown as yellow spheres with the name of each gene indicated. Interactions in Figs 2 and 3 are shown as black lines (from literature), solid green lines (from library screens in independent matings), dashed green lines (from library screens in one mating), purple (from array screens) and blue lines (from literature and screens). Arrows point away from the protein used as the binding-domain clone when the interaction was identified. Grey nubs indicate other proteins that interact with that protein but have not been expanded. **b**, Same pathway shown using the homologue viewer; the pathway can be rotated and homologous proteins in human, mouse, rat, *Drosophila*, *Caenorhabditis elegans* and *Escherichia coli* can be displayed. Known interactions between proteins in other species can be viewed: for this pathway interactions between the human proteins hMad3/hBub3, hBub3/hBub1, hBub1/hMad1, hBub3/hMad1 and hCDC20/hMad2, shown in black, are reported in the literature^{39–41}. The distance of each species protein icon (shown in key) from the yeast proteins (shown in yellow) represents the amount of overall similarity between the species. The size of the protein icon in each corresponding species indicates the amount of homology with the specific yeast protein. As the human homologues are highlighted in this example, their gene names are shown.

in mammalian cells. Screens of the three Sm proteins identified other Sm proteins (Table 2, Fig. 3b): the D1 homologue Lsm2 binds B (Lsm1), D2 (Smd2), E (Lsm5), F (Lsm6) and G (Lsm7) homologues; the D3 homologue Lsm4 binds B, F and G homologues and Lsm8; and Lsm8 binds D1, E, F and G homologues. These results support a proposed model¹⁹ based on crystallographic, biochemical and genetic data, but also include interactions not predicted by the model and which might reflect exchangeability of proteins within the complex, additional contacts within or between subcomplexes, or bridging effects in the two-hybrid assay.

In yeast, diverse cyclins bind to Cdc28 in a coordinated manner to modulate its kinase activity during the cell cycle. The B-type cyclins are critical in the induction of bipolar mitotic spindle formation²⁰. Each of the B-type cyclins, Clb1, Clb2 and Clb3, has been observed to be in a complex with Cks1 and Cdc28. Our observation of two-hybrid interactions between Cks1 and each of Clb1, Clb2 and Clb3 indicates that the kinase activity of Cdc28 could be regulated by cyclin Bs through their interaction with Cks1 (Fig. 3c).

Third, novel interactions that connect biological functions into larger cellular processes can be gleaned from our screens. The Sm proteins Lsm2, Lsm4 and Lsm8 identified ribosomal protein S28 (producing a signal with both Rps28a and Rps28b, the nearly identical copies of this protein), which may reflect an unusual

involvement of Rps28 in splicing, or of the snRNP proteins in translation or ribosome biogenesis (Fig. 3b). Unexpectedly, a characterization of the mammalian spliceosome complex by two-dimensional gel separation and mass spectrometry identified a different ribosomal protein, Rps4x, as a previously unknown spliceosome-associated protein²¹. The three Sm proteins also interacted with Dcp1, a messenger RNA-decapping enzyme, consistent with the role of Lsm1 (Spb8) in decapping²². Finally, a screen with Dcp1 found Rps28b and thus provides further evidence for a functional interplay between these proteins. Both Lsm2 and Lsm8 identified Mtr3, implicated in mRNA transport²³, suggesting another possible link between splicing and other processes.

The meiosis-specific protein Msh5 is required for the resolution of crossovers during meiosis²⁴. Meiotic recombination is initiated by double-strand breaks (DSBs), a prerequisite to crossover formation that is resolved in a structure called the synaptonemal complex. Mre11 is part of a complex that participates in DSB formation²⁵. It is also known that Tid3 helps form the spindle pole body and interacts with Dmc1 (ref. 26), a protein that is required for the formation of the synaptonemal complex. We observed Msh5 to interact with both Mre11 and Tid3 (Fig. 3d). These novel associations tie DSB formation and the resolution of crossovers with Msh5 as the linking protein.

Discussion

We have detected novel interactions for proteins previously screened by other workers who used the two-hybrid assay with activation-domain libraries of randomly generated inserts. Some of these new partners may reflect the comprehensive nature of the array and library approaches or the requirement for a full-length protein to detect some interactions. However, most of our data represent new potential interactions for proteins that have not been previously searched in the two-hybrid assay. Of the new interactions, many seem credible on the basis of genetic or other criteria, whereas the relevance of others cannot be easily assessed. However, some reproducible two-hybrid signals are unlikely to reflect true interactions, based on the known properties of the proteins involved. Thus, in the absence of other data, the set of proteins derived from each of these screens should be viewed as potential positives, serving to motivate other experiments that confirm or eliminate particular proteins as plausible interactors.

As part of these studies, we have developed a protein array approach as a new method for systematic genome-wide analysis. Arrays of biomolecules possess unique advantages for the handling and investigation of multiple samples. They provide a fixed location for each element such that those scoring positive in an assay are immediately identified; they have the capacity to be comprehensive and of high density; they can be screened by high-throughput robotic procedures using small volumes of reagents; and they allow the comparison of each assay value with the results of many other identical assays. Moreover, chemically pure protein arrays can be constructed by generating proteins fused to an affinity tag and recovering them by cell lysis and affinity purification⁷. The yeast activation-domain array and library described here also allow the detection *in vivo* of nucleic-acid-protein and small-molecule-protein interactions, through the use of hybrid molecules^{27–31}, in a manner similar to that for protein-protein interactions.

The publication of the complete genome sequence of *Caenorhabditis elegans* and the escalating efforts to complete the sequences of other genomes increase the need for high-throughput functional studies. The studies described here represent the first comprehensive biological screens that use the complete set of predicted ORFs from a eukaryotic organism. Both of the approaches could be scaled up for larger sets of proteins, such as those encoded by *Caenorhabditis elegans* or *Drosophila melanogaster*. The high-throughput library approach is reasonable to employ in order to complete a screen of all encoded ORFs for either of these organisms; however, the array approach, while much more time- and labour-intensive,

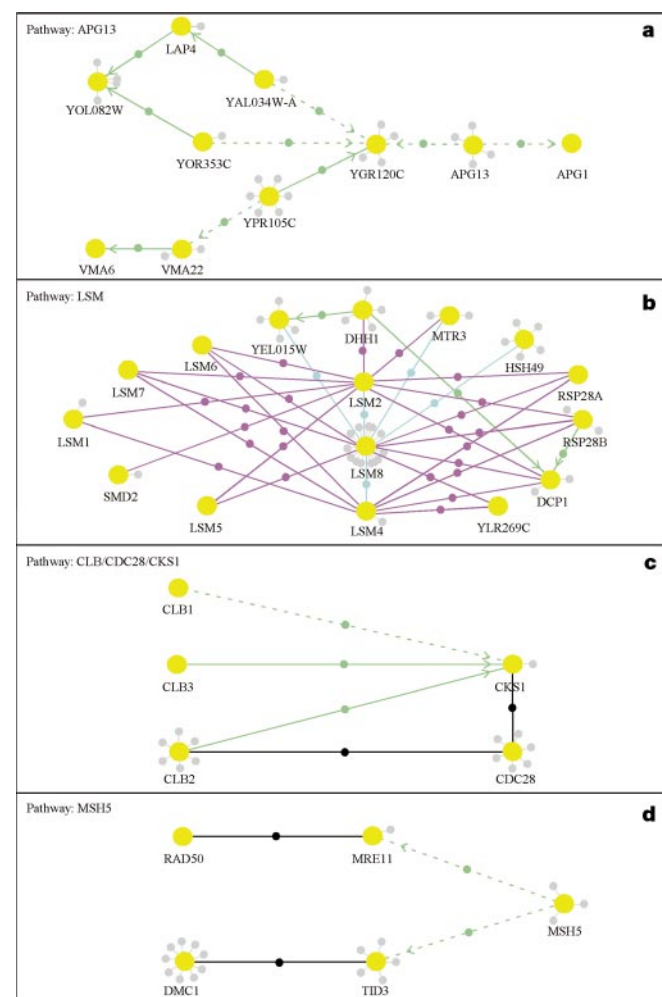


Figure 3 Expanded pathways shown using the software as described in the text. **a**, Autophagy pathway illustrating potential novel interactions that place functionally unclassified proteins in a biological context. **b**, Potential interactions identified by screens of the Sm motif-containing proteins Lsm2, Lsm4 and Lsm8. **c**, The Clb/Cdc28/Cks1 complex shows novel interactions between proteins involved in the same biological function. **d**, The Msh5 pathway illustrates novel interactions that link biological functions together into greater cellular processes.

would probably provide more positives. The bioinformatics platform we have developed can incorporate new data sets as they become available and compare results across species to identify conserved interactions. Systematically applying multiple high-throughput strategies should increase our understanding of yeast and other eukaryotic organisms. □

Methods

Gap-repair cloning

We constructed transformants containing activation-domain hybrids by recombination³² of the linearized vector pOAD⁸ with PCR fragments corresponding to each of the yeast ORFs⁸. The DNA-binding domain vector pOBD2 was constructed by introducing 48 base pairs from pOAD⁸, encoding residues 866–881 of the Gal4 activation domain, into pOBD⁸ immediately 3' to the DNA that encodes residue 147 of the Gal4 DNA-binding domain (S. McCraith and S.F., unpublished). This plasmid provides activation-domain sequences to allow cloning by recombination of the identical PCR fragments used to construct activation-domain hybrids. Transformation was carried out in a 96-well format using the lithium acetate procedure³³. Yeast media were prepared as described³⁴.

Generation of the array. After transformation, cells were plated on 35-mm synthetic plates without leucine. The yeast recipient for the activation-domain hybrid plasmids was PJ69-4a (ref. 9), which is *MATa*. Two colonies from each transformation plate were pooled, cultured in liquid-leucine medium and transferred to solid-leucine medium in OmniTrays (Nalge Nunc International) by a Biomek 2000 Laboratory Automation Workstation (Beckman). We constructed an isogenic *MATα* derivative by switching the PJ69-4a strain using a plasmid carrying the *HO* gene. Selection for transformants carrying DNA-binding domain hybrids used synthetic plates without tryptophan.

Collections for the library screens. Five microlitres from individual transformations were grown on selective medium lacking leucine or tryptophan for two days at 30 °C. Patches of transformants were manually transferred into individual wells on micro-assay plates for further use. The yeast recipients were YULH (*MATa ura3-52 trp1 lys2 his3 leu2 gal4 gal80 GAL1-URA3 GAL1-lacZ*) for the Gal4 DNA-binding domain fusion in pOBD2 and N106r (*MATα ura3-52 his3 ade2 trp1 leu2 gal4 gal80 cyh2 lys2::GAL1-HIS3 ura3::GAL1-lacZ*) for the Gal4 AD fusion in pOAD.

Screening procedure

Array screening. Transformants of the *a* and *α* reporter strains were mated on YEPD plates for 2–3 days at 30 °C by transferring ~1 ml of an overnight culture of the *MATα* strain expressing a DNA-binding domain hybrid onto each of 16 OmniTrays using a 384-pin High Density Replicating Tool (Beckman), and then pinning the activation-domain array transformants of the *MATa* strain onto the same positions. Diploids were selected by transfer with the replicating tool to medium without leucine and tryptophan, followed by 2–3 days of further growth. For the two-hybrid selection, the diploids were transferred to medium without leucine, tryptophan and histidine supplemented with 3 mM 3-amino-1,2,4-triazole and scored after two weeks of growth at 30 °C. Further details about the array screening procedure are available at <http://depts.washington.edu/sfields/>.

Library screening. Mating reactions were performed on 96-well filter plates (Millipore MAHV S45) by mixing 10⁷ *MATa* cells (Gal4 DNA-binding domain fusion) with 5 × 10⁶ *MATα* cells (activation domain library) from liquid cultures in complete medium (YPAD). After filtration, the 96-well filter plates were incubated overnight at 30 °C on rectangular YPAD solid medium plates. We collected cells from the mating mixes from each filter with sterile water. A semi-automated Zymark work station was used throughout the cloning and screening procedures. Diploids containing potential interactors were selected for 4 days at 30 °C on medium lacking leucine, tryptophan and uracil, and simultaneously screened for lacZ expression by the addition of X-gal. Each mating generated 5 × 10⁵ to 10⁶ original diploids per well, suggesting that the library was covered 80–160 times. Up to 12 blue colonies were picked per mating, generating a collection of 96-well plates of diploid clones as the final product of the screening process. The activation-domain fusion plasmids were submitted for PCR amplification and sequencing to identify the yeast ORF. A total of 8,676 blue colonies were picked from the screens: 6,909 (80%) passed PCR, sequencing, vector trimming and 6,215 (72%) passed interaction quality control. The resulting sequences were compared with the yeast sequence database using Blast2 (ref. 35). Sample handling and manipulation during the screens was tracked by computer and data analysis was carried out using GeneScape, web-based software developed at CuraGen.

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Table 2 List of the 957 interactions identified in both two-hybrid screens

BD fusion	AD fusion	Source	BD fusion	AD fusion	Source	BD fusion	AD fusion	Source
ADE2 (nm)	ADE2 (nm)	*	CYP2 (cs,vt)	JSN1 (st)	*	INO4 (li,p2)	HCS1 (ds)	*
ADE2 (nm)	RAD18 (ri)	*	CYS4 (aa)	YCR086W (me)	*	INO4 (li,p2)	INO2 (li,mi)	*,‡
ADE2 (nm)	SED4 (vt)	*	DAL3 (om)	DAL3 (om)	*	INO4 (li,p2)	YMR317W (un)	*
ADE2 (nm)	YBR134W (un)	*	DAL82 (om,p2)	DAL82 (om,p2)	*	INO4 (li,p2)	YNL279W (un)	*
ADE6 (nm)	YLR386W (un)	*	DAM1 (mi)	DUO1 (un)	*,‡	INO80 (un)	GDS1 (un)	*
ADE8 (nm)	MED4 (p2)	*	DCP2 (p2)	DCP1 (rt)	*	INO80 (un)	NHP10 (un)	*
ADE8 (nm)	SOH1 (un)	*	DCP2 (p2)	YEL015W (un)	*	IPK1 (li,nc)	RPS28B (ps)	*
ADE8 (nm)	YCR063W (un)	*	DHH1 (rp)	DCP1 (rt)	*	IPK1 (li,nc)	STE24 (pm)	*
ADH2 (cm)	YFL042C (un)	†	DHH1 (rp)	YEL015W (un)	*	IPK1 (li,nc)	YLR323C (un)	*
AHC1 (ch,p2)	YCR082W (un)	*	DIB1 (mi,rp,rs)	PRP6 (rp,rs)	*,f	IPK1 (li,nc)	YOR078W (un)	*
AIP1 (st)	APC9 (cc,mi,pd)	*	DIG1 (di)	DIG2 (di)	*	IRE1 (cs,om,si)	YDR532C (un)	†
ALR1 (sm)	GCD7 (ps)	*	DMC1 (me,rc)	DMC1 (me,rc)	*,‡	ISA1 (un)	RPS26A (ps)	*
ALR1 (sm)	PGD1 (p2)	*	DNA43 (ds)	MCM6 (ds)	*,‡	ISA1 (un)	RPS26B (ps)	*
ALR1 (sm)	YGL024W (un)	*	DUO1 (un)	BIM1 (cc,mi)	*	ISC10 (me)	TRP5 (aa)	*
ALR1 (sm)	YNL086W (un)	*	DUO1 (un)	YDR016C (un)	*	ISU2 (un)	YPL088W (un)	*
APG12 (pd,pm,vt)	AUT1 (cs,pt)	†	EBB1 (un)	DCP1 (rt)	*	KAP104 (nc)	YNR069C (un)	†
APG12 (pd,pm,vt)	SAP18 (pd)	*	EBB1 (un)	GAL83 (cm)	*	KAR4 (mr)	MUM2 (me)	*
APG13 (pd,vt)	APG1 (me,pd,vt)	*	EBB1 (un)	MAD2 (cc,mi)	*	KCS1 (p2)	BMH2 (di,si)	*
APG13 (pd,vt)	PUP2 (pd)	*	EBB1 (un)	MUM2 (me)	*	KEL1 (cp,mr)	CAF17 (p2)	*
APG13 (pd,vt)	SEC35 (vt)	*	EBB1 (un)	STD1 (cm,cs,sm)	*	KEL1 (cp,mr)	STD1 (cm,cs,sm)	*
APG13 (pd,vt)	YNL086W (un)	*	ECI2 (li)	ECI1 (li)	*	KEL1 (cp,mr)	YMR181C (un)	*
APG5 (pd,vt)	APG12 (pd,pm,vt)	*,†,¶,f	ECM19 (wm)	TAF61 (p2)	*	KGD2 (cm)	SMT3 (pm)	*
APG5 (pd,vt)	SAP18 (pd)	*	ECM31 (wm)	ECM31 (wm)	*	KIN1 (un)	YOL082W (un)	*
APG7 (di,pd,pt)	APG12 (pd,pm,vt)	*,‡	ENT3 (un)	YOR111W (un)	*	KRR1 (un)	MCM6 (ds)	*
APG7 (di,pd,pt)	AUT1 (cs,pt)	*	EST1 (ch,ds)	MCM6 (ds)	†	KSS1 (mr,si)	STE7 (di,mr)	†,‡
APG7 (di,pd,pt)	AUT7 (me,pd,vt)	*	EST1 (ch,ds)	SPT23 (ch)	†	LAP4 (pd)	LAP4 (pd)	*,f
APM1 (vt)	APL2 (vt)	*,f	EST1 (ch,ds)	URE1 (aa,om,p2)	†	LAP4 (pd)	YOL082W (un)	*
APM2 (vt)	APL2 (vt)	*	EST1 (ch,ds)	YAL028W (un)	†	LCP5 (rp)	BFR2 (pt)	*
ARH1 (en)	GDC39 (cc,p2)	*	EST1 (ch,ds)	YGR160W (un)	†	LEU4 (aa)	YKL183W (un)	*
ARH1 (en)	GIF1 (cc)	*	EST1 (ch,ds)	YJL019W (un)	†	LOS1 (nc)	RPC25 (p3)	†
ARP10 (st)	ARP1 (mi)	*	EST1 (ch,ds)	YMD8 (sm)	†	LPP1 (li)	SHE2 (cp,di)	*
ATP20 (en,sm)	SHE2 (cp,di)	*	FAP1 (un)	MRE11 (wm,chr,ri,me,rc)	*	LRS4 (p1)	YCR086W (me)	*
BEM4 (cp,si)	SCS3 (li)	*	FAP1 (un)	SHE2 (cp,di)	*	LSM2 (rp,rs)	DCP1 (rt)	†
BEM4 (cp,si)	YIL163C (un)	*	FAR3 (cc,mr)	YFR008W (un)	*	LSM2 (rp,rs)	DHH1 (rp)	†
BEM4 (cp,si)	YLR049C (un)	*	FMN1 (om)	YDR398W (un)	*	LSM2 (rp,rs)	LSM1 (rp,rs)	†,‡
BMH2 (di,si)	BOP3 (un)	*	FOL2 (om)	FOL2 (om)	*	LSM2 (rp,rs)	LSM5 (rp,rs)	†,‡
BMH2 (di,si)	ECM13 (wm)	*	FPR1 (un)	HOM3 (aa)	*,‡	LSM2 (rp,rs)	LSM6 (rp,rs)	†,‡
BUB2 (cc)	DDI1 (vt)	†	FPR1 (un)	YMR087W (un)	†	LSM2 (rp,rs)	LSM7 (rp,rs)	†,‡
BUB2 (cc)	GIC1 (cp,mr)	†	FUI1 (nm,sm)	RPN3 (pd)	*	LSM2 (rp,rs)	MTR3 (rp)	†
BUB2 (cc)	YGR153W (un)	†	FUN20 (rs,rp)	YLR345W (cm)	*	LSM2 (rp,rs)	RPS28A (ps)	†
BUB2 (cc)	YNL335W (un)	†	FUN20 (rs,rp)	YPL151C (un)	*	LSM2 (rp,rs)	RPS28B (ps)	†
BUB3 (cc)	MAD3 (cc)	*	FUR1 (nm)	APG13 (pd,vt)	*	LSM2 (rp,rs)	SMD2 (ch,ds,rp,rs)	†,‡
BUD6 (cp)	YGL015C (un)	*	FZF1 (om,p2)	CKB2 (p3)	*	LSM4 (rp,rs)	CKB2 (p3)	†
CAF20 (ps)	CDC33 (cc,ps)	*,f	FZF1 (om,p2)	PHO12 (ph)	*	LSM4 (rp,rs)	DCP1 (rt)	†
CAR1 (aa,om)	CAR1 (aa,om)	†	FZF1 (om,p2)	TFC4 (p3)	*	LSM4 (rp,rs)	LSM1 (rp,rs)	†,‡
CAR2 (aa,om)	SNF6 (ch,p2)	*	GCD7 (ps)	YPL070W (un)	*	LSM4 (rp,rs)	LSM6 (rp,rs)	†,f
CAR2 (aa,om)	YGR010W (un)	*	GCN3 (ps)	GCN3 (ps)	*	LSM4 (rp,rs)	LSM7 (rp,rs)	†,f
CAR2 (aa,om)	YLR328W (nm)	*	GCN5 (ch,p2,pm)	ADA2 (p2)	*,‡	LSM4 (rp,rs)	LSM8 (rp,rs)	†,f
CDC13 (ch)	STN1 (ch)	†,‡	GDH1 (aa)	LSM1 (rp,rs)	*	LSM4 (rp,rs)	RPS28A (ps)	†
CDC20 (mr,mi)	MAD2 (cc,mi)	*,‡	GFD1 (un)	NAB2 (nc,rp)	*	LSM4 (rp,rs)	RPS28B (ps)	†
CDC33 (cc,ps)	CAF20 (ps)	*,f	GIM5 (st,pf)	YKE2 (st)	*,f	LSM4 (rp,rs)	YLR269C (un)	†
CDC42 (cp,st,mr)	BEM4 (cp,si)	†,‡	GIS4 (un)	CUP2 (cs,p2)	*	LSM8 (rp,rs)	DCP1 (rt)	†
CDC42 (cp,st,mr)	RDI1 (un)	*	GLC7 (cm,cs,me,mi)	BDF2 (ch)	†	LSM8 (rp,rs)	DSS4 (vt)	†
CDC43 (pm)	RAM2 (mr,pm)	*,f	GLC7 (cm,cs,me,mi)	BN14 (wm,ck)	†,*	LSM8 (rp,rs)	HSH49 (rp,rs)	†,‡
CDC53 (aa,cc,pd)	CDC7 (ch,ri,ds,me,rc)	†	GLC7 (cm,cs,me,mi)	GIF1 (me)	†,‡	LSM8 (rp,rs)	LSM2 (rp,rs)	†,‡
CDC53 (aa,cc,pd)	SKP1 (aa,cc,ch,pd)	†,f	GLC7 (cm,cs,me,mi)	MHP1 (un)	†	LSM8 (rp,rs)	LSM5 (rp,rs)	†,‡
CDC53 (aa,cc,pd)	YLR100W (un)	†	GLC7 (cm,cs,me,mi)	PAN1 (cp,st,vt)	†	LSM8 (rp,rs)	LSM6 (rp,rs)	†,‡
CDC53 (aa,cc,pd)	YLR128W (un)	†	GLC7 (cm,cs,me,mi)	REF2 (rp)	†	LSM8 (rp,rs)	LSM7 (rp,rs)	†,‡
CDC53 (aa,cc,pd)	YLR368W (un)	†,†,¶	GLC7 (cm,cs,me,mi)	SCD5 (vt)	†,‡	LSM8 (rp,rs)	MTR3 (rp)	†
CDC53 (aa,cc,pd)	ZIP2 (me,rc)	†	GLC7 (cm,cs,me,mi)	YDR130C (un)	†	LSM8 (rp,rs)	RPS28A (ps)	†
CDC7 (ch,ri,ds,me,rc)	ARG3 (aa,om)	*	GLC7 (cm,cs,me,mi)	YDR412W (un)	†	LSM8 (rp,rs)	RPS28B (ps)	†
CDC7 (ch,ri,ds,me,rc)	DHH1 (rp)	*	GLC7 (cm,cs,me,mi)	YHR100C (un)	†	LSM8 (rp,rs)	YEL015W (un)	†,‡
CDC7 (ch,ri,ds,me,rc)	PTM1 (un)	*	GLC7 (cm,cs,me,mi)	YOR315W (un)	†	LSM8 (rp,rs)	YLR269C (un)	†
CDC7 (ch,ri,ds,me,rc)	TEL2 (ch)	*	GLG2 (cm)	GLG2 (cm)	*	LST7 (vt)	PUT3 (aa)	*
CDC7 (ch,ri,ds,me,rc)	YCR022C (un)	*	GLK1 (cm)	ARGR2 (aa)	*	LYS5 (aa)	FZF1 (om,p2)	*
CDC7 (ch,ri,ds,me,rc)	YCR050C (un)	*	GLK1 (cm)	FIG1 (mr)	*	MAK3 (pm)	MAK10 (en,ot)	*
CDC7 (ch,ri,ds,me,rc)	YEL023C (un)	*	GPD2 (cm)	GNA1 (cc,wm)	*	MAK31 (rt)	MAK10 (en,ot)	*
CDC7 (ch,ri,ds,me,rc)	YFR057W (un)	*	GRX3 (cs)	RCS1 (sm)	*	MCM16 (mi)	MCM22 (ch)	*
CDC7 (ch,ri,ds,me,rc)	YNR048W (un)	*	GRX5 (cs)	YIL105C (un)	*	MCM22 (ch)	MCM16 (mi)	*
CDC7 (ch,ri,ds,me,rc)	YOR006C (un)	*	GRX5 (cs)	YNL047C (un)	*	MEC3 (cc)	SAP18 (pd)	*
CDD1 (nm)	CDD1 (nm)	*	GSB1 (me)	YHB1 (un)	*	MED11 (p2)	SRB6 (p2)	*
CHK1 (cc)	GFD1 (un)	*	GSP1 (nc)	MOG1 (nc)	*,‡	MED7 (p2)	MED4 (p2)	*,f
CHK1 (cc)	GSY2 (cm)	*	GSP2 (nc)	MOG1 (nc)	*	MER1 (rp,rc)	MRP8 (ps)	*
CIN4 (mr,mi)	GYP1 (vt)	†	GTR1 (sm)	GTR1 (sm)	*	MET18 (aa,ri,p2)	MED11 (p2)	†
CIN4 (mr,mi)	YBR284W (un)	†	GZF3 (om,p2)	HDA1 (ch,pm)	*	MET30 (aa,om,pd)	HDR1 (me)	†
CIT2 (en)	CIT2 (en)	†,*	HAP2 (cm,en,p2)	HAP3 (p2)	*,‡	MET30 (aa,om,pd)	MNS1 (pm)	†
CIT2 (en)	MHP1 (un)	†	HAP2 (cm,en,p2)	HAP5 (p2)	*,‡	MET30 (aa,om,pd)	SRL2 (un)	†
CKA2 (p2)	CKB2 (p3)	*	HAP3 (p2)	HAP5 (p2)	*,‡	MET30 (aa,om,pd)	YBR270C (un)	†
CLB1 (cc)	CKS1 (cc)	*	HEX3 (cm)	SMT3 (pm)	*,†,¶	MET30 (aa,om,pd)	YEL015W (un)	†
CLB2 (cc)	CKS1 (cc)	*	HEX3 (cm)	YER116C (un)	*	MET30 (aa,om,pd)	YJL058C (un)	†
CLB2 (cc)	FPR1 (un)	*	HIS3 (aa)	HIS3 (aa)	*	MET31 (aa,p2)	GCN4 (aa,nm,p2)	*
CLB2 (cc)	MUS81 (ri)	*	HOR2 (cm,cs)	YPL201C (un)	*	MIG1 (cm,p2,si)	YPL025C (un)	†
CLB2 (cc)	YDR412W (un)	*	HPR5 (ri)	GDS1 (un)	*	MKK2 (cs)	SLT2 (cs,wm,si)	*,‡
CLB2 (cc)	YHR035W (un)	*	HPR5 (ri)	MEL1 (cm)	*	MOB2 (cc)	YOL036W (un)	*
CLB2 (cc)	YNR022C (un)	*	HPR5 (ri)	SMT3 (pm)	*	MOG1 (nc)	GSP2 (nc)	*
CLB3 (cc)	CKS1 (cc)	*	HPR5 (ri)	YDR078C (un)	*	MPD2 (pf)	MSR1 (ps)	*
CLN3 (cc)	MAD3 (cc)	*	HPR1 (rp)	NAB2 (nc,rp)	*	MPD2 (pf)	YLR312C (un)	*
CNA1 (cp,mr,si,sm)	YNL047C (un)	*	HRR25 (ri)	ADY1 (un)	*	MRP8 (ps)	MRP8 (ps)	*
CNB1 (om,sm)	YMR211W (un)	†	HRT1 (pm)	YAK1 (cc,cs)	*	MRP8 (ps)	SMP2 (wm,en)	*
CNS1 (un)	STP1 (rp,sm)	†	HRT1 (pm)	YIL001W (un)	*	MSB2 (cp)	CDC36 (cc,p2)	*
COF1 (st,vt)	ACT1 (cp,st,vt)	†,f	HRT1 (pm)	YJL200C (un)	*	MSB2 (cp)	MAD2 (cc,mi)	*
CPA1 (aa)	CKB2 (p3)	*	HSH49 (rp,rs)	YBR103W (ch)	†	MSB2 (cp)	MAD3 (cc)	*
CPR6 (pf)	HYR1 (cs)	*	HST3 (ch)	SFP1 (cc,nc)	*	MSB2 (cp)	NIP7 (rp)	*
CRN1 (un)	SKP1 (aa,cc,ch,pd)	*	HTA1 (ch,p2)	NAP1 (ch)	†,*	MSB2 (cp)	TID3 (mi)	*
CSE2 (cc,mi,p2)	MED4 (p2)	*,f	HTA3 (ch)	YJL019W (un)	†	MSH5 (me,rc)	HSL7 (cc)	*
CTF13 (cc,ch,mi)	AQY2 (sm)	†	IME4 (me,p2)	MUM2 (me)	*	MSH5 (me,rc)	MRE11 (wm,chr,ri,me,rc)	*
CTF13 (cc,ch,mi)	YOR359W (un)	†	INO4 (li,p2)	APL2 (vt)	*	MSH5 (me,rc)	PGD1 (p2)	*



BD fusion	AD fusion	Source	BD fusion	AD fusion	Source	BD fusion	AD fusion	Source
MSH5 (me,rc)	TID3 (mi)	*	PUT4 (aa,sm)	MTF1 (mt)	*	SME1 (ch,ds,rp,rs)	SMX3 (ch,ds,rp,rs)	*,‡
MSH5 (me,rc)	YGL170C (cm)	*	PUT4 (aa,sm)	YCR045C (pd)	*	SMI1 (wm)	BAS1 (aa,nm,p2)	*
MSN5 (mr,nc)	HMO1 (ch)	†	PUT4 (aa,sm)	YJL084C (un)	*	SMK1 (wm,di)	CAK1 (cc,mr,me)	*
MSN5 (mr,nc)	SEC9 (vt)	†	PUT4 (aa,sm)	YLR294C (un)	*	SMT3 (pm)	UBC9 (cc,pd,pm)	†,f
MSN5 (mr,nc)	SWI5 (cc,ch)	*	RAD10 (ri,rc)	GCD1 (ps)	†	SMX3 (ch,ds,rp,rs)	SMD2 (ch,ds,rp,rs)	*
MSN5 (mr,nc)	YOR359W (un)	†	RAD10 (ri,rc)	YCR086W (me)	†	SNF1 (cm,cs,li,si)	GAL83 (cm)	†,*,‡
MSN5 (mr,nc)	YPR008W (un)	†	RAD17 (cc,ri)	MEC3 (cc)	*,f	SNF1 (cm,cs,li,si)	SIP2 (cm)	†,‡
MTR3 (rp)	RRP42 (rp,rt)	*	RAD4 (ri)	RAD23 (ri)	†,‡	SNF1 (cm,cs,li,si)	YNL218W (ds)	†
MTW1 (un)	LAP4 (pd)	*	RAD4 (ri)	RPS31 (ca,ps)	†	SNF4 (si)	COQ5 (en,om)	†
MTW1 (un)	NUP49 (nc)	*	RAD51 (ri,rc)	SAP1 (ch)	†	SNF4 (si)	GAL83 (cm)	†,*,‡
MTW1 (un)	SEC35 (vt)	*	RAD51 (ri,rc)	UBC9 (cc,pd,pm)	†	SNF4 (si)	YDL214C (un)	†
NAB2 (nc,rp)	GON3 (ps)	*	RAD51 (ri,rc)	YMR233W (un)	†	SNF4 (si)	YJL114W (un)	†
NDC1 (mi)	ASM4 (ds,nc)	*	RAD51 (ri,rc)	YPL238C (un)	†	SNF4 (si)	YJR083C (un)	†
NDC1 (mi)	NUP53 (nc)	*	RAD51 (ri,rc)	YPR011C (sm)	†	SNF4 (si)	YMR291W (un)	†
NHP10 (un)	YER092W (un)	*	RAD55 (ri,mi,rc)	RAD51 (ri,rc)	*,‡	SNO1 (cs)	SNZ1 (cs)	*,‡
NIC96 (nc)	NUP53 (nc)	*	RAD6 (ch,ri,me,pd,pm,rc)	RAD18 (ri)	*,f	SNO1 (cs)	SNZ3 (cs)	*
NIC96 (nc)	SEC35 (vt)	*	RAM1 (mr,pm)	RAM2 (mr,pm)	*,f	SNO3 (cs)	SNZ1 (cs)	*
NIF3 (p2,p2,pm)	NIF3 (p2,p2,pm)	*	RCK1 (un)	HOG1 (cs,si)	*	SNO3 (cs)	SNZ3 (cs)	*
NIP1 (ps)	SUI1 (ps)	*,‡	RGA1 (cp,mr)	YHL042W (un)	*	SNP1 (rp,rs)	KIN3 (un)	†
NIP1 (ps)	YNL047C (un)	*	RGA1 (cp,mr)	YJL185C (un)	*	SNU23 (rp,rs)	PRP38 (rs)	*,f
NIP1 (ps)	YOR284W (un)	*	RGR1 (p2)	PEX19 (li)	*	SNZ1 (cs)	SNO1 (cs)	*,‡
NIP100 (mi,nc)	ARP1 (mi)	*,f	RHO1 (cc,si)	BEM4 (cp,si)	†,‡	SPC19 (mi)	TID3 (mi)	*
NIP100 (mi,nc)	TID3 (mi)	*	RHO4 (cp)	BEM4 (cp,si)	†,‡	SPC34 (ck)	JSN1 (st)	*
NIP100 (mi,nc)	YJL184W (un)	*	RIM101 (di,me,p2)	ZAP1 (p2)	*	SPC34 (ck)	SPC19 (mi)	*
NPL4 (nc)	UDF1 (pd)	*	RIM11 (me)	IME1 (me,p2)	*,‡	SPO1 (me)	YBR250W (un)	†
NPL6 (nc)	RSC8 (ch,p2)	*	RLF2 (ch)	MSI1 (ch,ri)	*,f	SPO11 (me,rc)	SKI8 (ri,rc)	†
NTH1 (nc,cs)	YLR270W (un)	*	RNP1 (rp)	YFR047C (aa,om)	*	SPO12 (di,me)	YCR086W (me)	†
NUF2 (mi)	PRS2 (aa,nm)	*	RPB10 (p1,p2,p3,ca)	CUP2 (cs,p2)	*	SPO13 (di,me)	ADY1 (un)	*
NUP157 (nc)	MAD2 (cc,mi)	*	RPC19 (p1,p3)	GTR1 (sm)	†	SPR28 (ck)	CDC11 (cp,wm,ck)	*
NUP157 (nc)	NUP53 (nc)	*	RPC19 (p1,p3)	YFR011C (un)	†	SPT2 (ch)	FOB1 (rc)	†
NUP157 (nc)	YEL015W (un)	*	RPC19 (p1,p3)	YHL018W (un)	†	SPT2 (ch)	MCM6 (ds)	*
NUP2 (nc)	HSP10 (p2)	†	RPC19 (p1,p3)	YLR266C (p2)	†	SRB5 (p2)	MED8 (p2)	*,f
NUP57 (nc)	NSP1 (nc)	*	RPC40 (p1,p3)	RPC19 (p1,p3)	*,†,‡,‡	SRB7 (p2)	MED4 (p2)	*
NUP57 (nc)	NUP49 (nc)	*	RPC40 (p1,p3)	YLR238W (un)	*	SRB7 (p2)	MED7 (p2)	*
NUP57 (nc)	TAF17 (p2)	*	RPC53 (p3)	YKR025W (un)	*	SRL2 (un)	SRL2 (un)	*
PAC1 (st)	YLR254C (un)	*	RPN8 (pd)	RPN8 (pd)	†,f	SRP1 (cc,nc)	ARG1 (aa,om)	*
PCF11 (rp)	ADA2 (p2)	†	RPN12 (pd)	XPT1 (nm)	*	SRP1 (cc,nc)	CAR1 (aa,om)	*
PCF11 (rp)	CLP1 (rp)	†,‡	RPN12 (pd)	YDR273W (un)	*	SRP1 (cc,nc)	DUT1 (nm)	*
PCF11 (rp)	RNA14 (rp)	†,‡	RPN5 (pd)	SPC19 (mi)	†	SRP1 (cc,nc)	FCY1 (nm)	*
PCF11 (rp)	RNA15 (rp)	†,‡	RPN5 (pd)	YDR179C (un)	†	SRP1 (cc,nc)	ICL1 (cm)	*
PCF11 (rp)	YLR423C (un)	†	RPN6 (pd)	GON4 (aa,nm,p2)	*	SRP1 (cc,nc)	MET17 (aa)	*
PCF11 (rp)	YLR424W (un)	†	RPP0 (ps)	RPP1A (ps)	†	SRP1 (cc,nc)	NIF3 (p2,pm)	*
PCF11 (rp)	YPR049C (un)	†	RPS26A (ps)	YLR435W (un)	*	SRP1 (cc,nc)	PHO13 (ph)	*
PCL10 (cm)	PHO85 (ph,si)	*,†,‡,‡	RPS26B (ps)	YLR435W (un)	*	SRP1 (cc,nc)	SHE2 (cp,di)	*
PCL2 (cc)	SWI5 (cc,ch)	*	RPS28B (ps)	DGP1 (rt)	*	SRP1 (cc,nc)	SOR1 (cm)	*
PCL6 (un)	YJL084C (un)	*	RPS28B (ps)	YBR094W (un)	*	SRP1 (cc,nc)	THI6 (om)	*
PCL6 (un)	YLR190W (un)	*	RPS8B (ps)	CKS1 (cc)	*	SRP1 (cc,nc)	TSA1 (cs)	*
PDB1 (cm,en)	YLR345W (cm)	*	RPS8B (ps)	GNA1 (cc,wm)	*	SRP1 (cc,nc)	XPT1 (nm)	*
PDR11 (sm)	HMO1 (ch)	*	RPT3 (pd)	AME1 (ck)	†	SRP1 (cc,nc)	YAP3 (un)	*
PEX7 (li,pt)	PEX18 (pt)	*,‡	RPT3 (pd)	RPT3 (pd)	†	SRP1 (cc,nc)	YFL061W (un)	*
PEX7 (li,pt)	PEX21 (pt)	*,‡	RPT3 (pd)	RPT4 (pd)	†,‡	SRP1 (cc,nc)	YGR024C (un)	*
PEX7 (li,pt)	POT1 (li)	*,‡	RPT3 (pd)	RPT5 (pd)	†,‡	SRP1 (cc,nc)	YMR226C (un)	*
PFK1 (cm)	UBP8 (pd)	†	RPT3 (pd)	YGR232W (un)	*	SRP101 (pt)	YMR163C (un)	*
PHO85 (ph,si)	CLG1 (un)	†,‡	RRN10 (p1)	YDL113C (un)	†	SSP1 (me)	GDS1 (un)	*
PHO85 (ph,si)	PCL2 (cc)	†,‡	RRN7 (p1)	RRN6 (p1)	*,‡	STE11 (mr,si)	STE50 (di,mr,si)	*,‡
PHO85 (ph,si)	PCL6 (un)	†,‡	RRN9 (p1)	RRN10 (p1)	*,†,‡,‡	STE12 (mr,p2,si)	DIG2 (di)	*
PHO85 (ph,si)	PCL7 (un)	†,‡	RSB1 (un)	YOL083W (un)	*	STE18 (cp,mr,si)	STE4 (mr)	*,‡
PHO85 (ph,si)	PCL8 (cm)	†,‡	RTA1 (un)	YHR134W (un)	*	STM1 (un)	YJR072C (un)	*
PHO85 (ph,si)	PCL9 (cc)	†,‡	RVS161 (cp,st,mr,vt)	RVS167 (cp,st,vt)	*,*,†,f	STT4 (li)	GDS1 (un)	*
PHO85 (ph,si)	SOR1 (cm)	†	RVS161 (cp,st,mr,vt)	YBR108W (un)	*	STT4 (li)	STD1 (cm,cs,sm)	*
PHO85 (ph,si)	YDL246C (cm)	†	SAE2 (me)	SAE2 (me)	*	SXM1 (nc)	YDR132C (un)	†
PHO85 (ph,si)	YNL201C (cm)	†	SAE2 (me)	YCR086W (me)	*	SXM1 (nc)	YOL070C (un)	†
PIB1 (vt)	YPL133C (un)	*	SAP4 (cc)	MAD2 (cc,mi)	*	SYF1 (rp,rs)	ISY1 (rp,rs)	*,‡
PKC1 (wm,si)	ZDS2 (cc)	*	SAP4 (cc)	MAD3 (cc)	*	SYF1 (rp,rs)	NTC20 (rp,rs)	*
PKH1 (si)	YHR207C (un)	*	SAP4 (cc)	PGI1 (cm)	*	SYF1 (rp,rs)	SYF2 (rp,rs)	*
PKH1 (si)	YIR044C (un)	*	SAP4 (cc)	YJL178C (un)	*	SYF3 (rp,rs)	NTC20 (rp,rs)	*
PKH1 (si)	YRF1-4 (ch)	*	SAP4 (cc)	YJL211C (un)	*	TAF40 (p2)	HMO1 (ch)	*
POM152 (nc)	IKS1 (un)	*	SAP4 (cc)	YMR181C (un)	*	TAF40 (p2)	TAF25 (p2)	*
POM152 (nc)	NUP53 (nc)	*	SAP4 (cc)	YOR062C (un)	*	TAF60 (p2)	GFD1 (un)	*
PPA2 (ph)	GON3 (ps)	*	SAP4 (cc)	YPR040W (un)	*	TAF60 (p2)	TAF17 (p2)	*,f
PPG1 (cm)	CPR6 (pf)	†	SEC14 (li,vt)	YDL001W (un)	*	TAH18 (un)	GCR2 (p2)	*
PPG1 (cm)	HSC82 (cs,ot)	†	SEC21 (vt)	YBR281C (un)	*	TAH18 (un)	GDH2 (aa,om)	*
PPG1 (cm)	PPT1 (un)	†	SED1 (st)	HEM13 (om)	*	TAH18 (un)	GDS1 (un)	*
PPT1 (un)	ADR1 (p2)	†	SHR3 (pf,sm,vt)	GNP1 (aa,sm)	*	TAH18 (un)	MED1 (un)	*
PRB1 (pd)	YML032CA (un)	*	SIN4 (p2)	CDC34 (pd)	*	TEM1 (mi)	DIG1 (di)	†
PRE10 (pd)	GNA1 (cc,wm)	*	SIN4 (p2)	CSE1 (mi,nc)	*	TEM1 (mi)	DMC1 (me,rc)	†
PRE3 (pd)	YLR386W (un)	*	SIN4 (p2)	GDS1 (un)	*	TEM1 (mi)	ECI1 (li)	†
PRE5 (pd)	SHE2 (cp,di)	*	SIN4 (p2)	MAD2 (cc,mi)	*	TEM1 (mi)	FIL1 (en,ps)	†
PRO3 (aa)	PRO3 (aa)	*	SIN4 (p2)	MAD3 (cc)	*	TEM1 (mi)	FOB1 (rc)	†
PRP11 (rp,rs)	PRP21 (rp,rs)	†,‡	SIN4 (p2)	PET191 (en)	*	TEM1 (mi)	HMO1 (ch)	†
PRP5 (rp,rs)	SNF11 (ch,p2)	*	SIN4 (p2)	PRP40 (rp,rs)	*	TEM1 (mi)	HSP10 (pf)	†
PRP6 (rp,rs)	DIB1 (mi,rp,rs)	†,*,f	SIN4 (p2)	QCR6 (en,sm)	*	TEM1 (mi)	KIN3 (un)	†
PRP6 (rp,rs)	UBC9 (cc,pd,pm)	†	SIN4 (p2)	YGR046W (un)	*	TEM1 (mi)	LAC1 (vt)	†
PRS5 (aa,nm)	PRS2 (aa,nm)	*,‡	SIN4 (p2)	YGR117C (un)	*	TEM1 (mi)	MNS1 (pm)	†
PSE1 (nc)	APL2 (vt)	†	SIP2 (cm)	SNF4 (si)	*,†,‡,f	TEM1 (mi)	SEC66 (pt)	†
PSE1 (nc)	HSP10 (pf)	†	SKP1 (aa,cc,ch,pd)	BDF1 (ch,me)	†	TEM1 (mi)	SMX3 (ch,ds,rp,rs)	†
PSE1 (nc)	KGB2 (cm)	†	SKP1 (aa,cc,ch,pd)	CDC4 (cc,pm)	†,*,f	TEM1 (mi)	SNZ2 (cs)	†
PSE1 (nc)	NUP57 (nc)	†,f	SKP1 (aa,cc,ch,pd)	CTF13 (cc,ch,mi)	†,f	TEM1 (mi)	SOR1 (cm)	†
PSE1 (nc)	SOR1 (cm)	†	SKP1 (aa,cc,ch,pd)	GRR1 (aa,cm,cc,pd)	†,f	TEM1 (mi)	WTM2 (me,p2)	†
PSE1 (nc)	WTM2 (me,p2)	†	SKP1 (aa,cc,ch,pd)	MET30 (aa,om,pd)	†,f	TEM1 (mi)	YCR087W (un)	†
PSE1 (nc)	YDL246C (cm)	†	SKP1 (aa,cc,ch,pd)	RUB1 (cc,pm,ps)	†	TEM1 (mi)	YDL246C (cm)	†
PSE1 (nc)	YER045C (un)	†	SKP1 (aa,cc,ch,pd)	SGT1 (mi,pd)	†,f	TEM1 (mi)	YEL015W (un)	†
PSE1 (nc)	YIR025W (un)	†	SKP1 (aa,cc,ch,pd)	YLR097C (un)	†	TEM1 (mi)	YHR198C (un)	†
PSE1 (nc)	YMR048W (un)	†	SKP1 (aa,cc,ch,pd)	YLR224W (un)	†	TEM1 (mi)	YJL058C (un)	†
PSE1 (nc)	YPL133C (un)	†	SKP1 (aa,cc,ch,pd)	YLR352W (un)	*	TEM1 (mi)	YJR056C (un)	†
PTC1 (rp,si)	NBP2 (ch)	*	SKP1 (aa,cc,ch,pd)	YLR368W (un)	†	TEM1 (mi)	YLR423C (un)	†
PTC2 (si)	YDR071C (un)	†	SLU7 (rp,rs)	YDL144C (un)	*	TEM1 (mi)	YNL218W (ds)	†



BD fusion	AD fusion	Source	BD fusion	AD fusion	Source	BD fusion	AD fusion	Source
TEM1 (mi)	YPL192C (un)	†	YDL012C (un)	YJL065C (un)	*	YHR083W (un)	SPC19 (mi)	†
TFA1 (p2)	TFA2 (p2)	*,f	YDL063C (un)	RPL5 (ps)	*	YHR083W (un)	YNL092W (un)	†
TFB1 (p2)	TFB1 (ri,p2)	*	YDL063C (un)	YRA1 (rp)	*	YHR105W (un)	AKR2 (vt)	†
TFB1 (ri,p2)	LAP4 (pd)	*	YDL071C (un)	GNA1 (cc,wm)	*	YHR105W (un)	KTR3 (pm)	†
TFB1 (ri,p2)	SEC35 (vt)	*	YDL071C (un)	YDR183W (un)	*	YHR105W (un)	YGL161C (un)	†
TFB1 (ri,p2)	YOL082W (un)	*	YDL071C (un)	YEL068C (un)	*	YHR105W (un)	YIF1 (un)	†
THI4 (ri,om)	THI4 (ri,om)	*	YDL071C (un)	YGR269W (un)	*	YHR105W (un)	YPL246C (un)	†
THI6 (om)	THI6 (om)	*	YDL071C (un)	YNL155W (un)	*	YHR111W (om)	YHR111W (om)	†
THI6 (om)	GNA1 (cc,wm)	*	YDL076C (un)	CHA4 (aa)	*	YHR115C (un)	YEL041W (un)	†
TOP1 (ch,mi)	YMR233W (un)	*	YDL089W (un)	YDL089W (un)	*	YHR115C (un)	YLR215C (un)	†
TPK3 (cc)	SRA1 (cc,cs,si)	*,f	YDL089W (un)	YLR324W (un)	*	YHR145C (un)	GCN4 (aa,nm,p2)	*
TPK3 (cc)	TPK3 (cc)	†	YDL089W (un)	YMR316CB (un)	*	YHR188C (un)	YJR015W (un)	†
TPO1 (sm)	CUP2 (cs,p2)	*	YDL110C (un)	PUT3 (aa)	*	YHR197W (un)	YLR423C (un)	†
TPS2 (cm,cs)	SBH2 (pt)	*	YDL110C (un)	YOR078W (un)	*	YHR204W (pm)	RPL30 (ps,rs,rt)	*
TPS2 (cm,cs)	YAR066W (un)	*	YDL113C (un)	YFL036W (un)	*	YHR204W (pm)	YER126C (un)	*
TRS31 (vt)	BET3 (vt)	*,f	YDL133W (un)	YDL001W (un)	*	YIL007C (pd)	ADY1 (un)	*
TRS31 (vt)	TRS20 (vt)	*,f	YDL146W (un)	YKL070W (un)	*	YIL008W (un)	YHR111W (om)	*
TRS33 (vt)	YOL082W (un)	*	YDL203C (un)	NDT1 (cc,p2)	*	YIL011W (un)	RAD14 (ri)	*
TSP1 (p2)	YMR316CB (un)	*	YDL203C (un)	YGR058W (un)	*	YIL065C (un)	JSN1 (st)	*
UBC6 (pd,pm)	MIR1 (en,sm)	†	YDL213C (un)	YFR032C (un)	†	YIL065C (un)	SFH1 (ch)	*
UBC6 (pd,pm)	YPL229W (un)	†	YDL216C (un)	YMR025W (un)	*	YIL074C (un)	YER081W (un)	*
UBC9 (cc,pd,pm)	UBC9 (cc,pd,pm)	†	YDL239C (un)	CHA4 (aa)	*	YIL074C (un)	YIL074C (un)	*
UBP5 (pm)	YBR059C (un)	*	YDL239C (un)	SPO21 (un)	*	YIL082W (un)	YGR024C (un)	*
ULA1 (pm)	UBA3 (pm)	*	YDL239C (un)	SSP1 (me)	*	YIL105C (un)	DMC1 (me,rc)	*
UME6 (me,om)	GDS1 (un)	*	YDL246C (cm)	SOR1 (cm)	*	YIL105C (un)	SHE2 (cp,di)	*
UME6 (me,om)	YOL082W (un)	*	YDL246C (cm)	YDL246C (cm)	*	YIL105C (un)	YNL047C (un)	*
URA3 (nm)	URA3 (nm)	†	YDR013W (un)	YDR489W (un)	*	YIL113W (un)	KNS1 (un)	†
URK1 (nm)	YDR020C (un)	*	YDR026C (un)	FOB1 (rc)	*	YIL113W (un)	SLT2 (cs,wm,si)	†
VAM7 (mf)	KTR3 (pm)	†	YDR032C (un)	STE50 (di,mr,si)	*	YIL132C (un)	SFH1 (ch)	*
VAM7 (mf)	SEC10 (vt)	†	YDR032C (un)	YDR032C (un)	*	YIL132C (un)	YLR322W (un)	*
VAM7 (mf)	SPC72 (me,mi)	†	YDR051C (un)	YDR051C (un)	*	YIL132C (un)	YLR376C (un)	*
VAM7 (mf)	STB2 (un)	†	YDR061W (ri)	YGR086W (me)	*	YIL151C (un)	YBL051C (rp)	*
VAM7 (mf)	VAM3 (mf,vt)	†,f	YDR063W (un)	ARC19 (cp,st)	†	YIL151C (un)	YDR140W (un)	*
VAM7 (mf)	YGL104C (sm)	†	YDR063W (un)	YGL239C (un)	†	YIL151C (un)	YPS4 (un)	*
VAM7 (mf)	YGL161C (un)	†	YDR070C (un)	GNA1 (cc,wm)	*	YIR005W (un)	SPT15 (p1,p2,p3)	†
VAM7 (mf)	YIF1 (un)	†	YDR071C (un)	YBR125C (un)	*	YIR005W (un)	YGL174W (un)	†,*
VAM7 (mf)	YJL151C (un)	†	YDR084C (un)	YGL161C (un)	*	YIR005W (un)	YMR057C (un)	†
VAM7 (mf)	YLR423C (un)	†	YDR084C (un)	YGL198W (un)	*	YJL048C (un)	SHE2 (cp,di)	*
VAM7 (mf)	YMR071C (un)	†	YDR115W (en,ps)	MRP8 (ps)	*	YJL112W (un)	DNM1 (vt)	*
VAM7 (mf)	YOL129W (un)	†	YDR128W (un)	SEC13 (sm,vt)	*	YJL160C (un)	YCR059C (un)	*
VAM7 (mf)	YOR292C (un)	†	YDR132C (un)	NMD3 (ca,rt)	*	YJR024C (cm)	YJR024C (cm)	*
VAM7 (mf)	YPL246C (un)	†	YDR132C (un)	YJL218W (un)	*	YJR072C (un)	YLR243W (un)	*
VAM7 (mf)	YPT7 (vt)	†	YDR179C (un)	YMR025W (un)	*	YJR072C (un)	YOR262W (un)	*
VIK1 (un)	YMR051C (un)	†	YDR200C (un)	FAR3 (cc,mr)	*	YJR136C (un)	YKL033W (un)	*
VMA22 (ca,sm)	VMA6 (sm)	*	YDR200C (un)	YNL127W (un)	*	YJU2 (un)	SYF1 (rp,rs)	†
VMA22 (ca,sm)	YDR469W (un)	*	YDR214W (un)	MAD2 (cc,mi)	*	YJU2 (un)	URE2 (aa,om,p2)	†
VPS17 (vt)	VPS5 (vt)	*	YDR215C (un)	DBP7 (rp)	*	YKE2 (st)	FAR3 (cc,mr)	*
VPS27 (vt)	YHL002W (un)	*	YDR215C (un)	SPT14 (wm,pm)	*	YKL002W (un)	SPC24 (mi)	*
VPS35 (vt)	CUP2 (cs,p2)	*	YDR255C (un)	RPC25 (p3)	*	YKL090W (un)	TBF1 (ch)	*
VPS36 (vt)	SNF8 (cm,si)	*	YDR267C (un)	YHR122W (un)	*	YKL090W (un)	YGR024C (un)	*
VPS4 (vt)	SNF7 (cm,vt)	*	YDR279W (un)	YLR154C (un)	*	YKL116C (un)	ASK10 (p2,si)	†
WSC3 (cs,wm)	PEX14 (li,pt)	*	YDR326C (un)	YER007CA (un)	*	YKL116C (un)	STE23 (mr,pm)	†
YAL036C (un)	YDR152W (un)	*	YDR326C (un)	YIL105C (un)	*	YKR007W (un)	YBR077C (un)	*
YAP5 (p2)	RCS1 (sm)	*	YDR348C (un)	YMR295C (un)	*	YKR011C (un)	CUP2 (cs,p2)	*
YAR003W (un)	YBR175W (un)	*	YDR357C (un)	SMX3 (ch,ds,rp,rs)	*	YKR022C (un)	YBL010C (un)	*
YAR003W (un)	YDR140W (un)	*	YDR400W (un)	YCR059C (un)	*	YKR022C (un)	YLR424W (un)	*
YAR014C (un)	GLC7 (cm,cs,me,mi)	*	YDR482C (un)	CAC20 (ps)	*	YKR060W (un)	YDR179C (un)	*
YAR031W (un)	APG12 (pd,pm,vt)	*	YDR482C (un)	SCW11 (wm)	*	YKR083C (un)	YKL052C (un)	*
YAR031W (un)	YCR030C (un)	*	YEL023C (un)	YDL011C (un)	*	YKR096W (un)	YBL051C (rp)	*
YBL101W-A (un)	ECM13 (wm)	*	YFR042W (un)	KRE6 (cm,wm)	*	YKR104W (sm)	RIB4 (om)	*
YBL101W-A (un)	URE2 (aa,om,p2)	*	YFR043C (un)	YDR489W (un)	*	YLL049W (un)	YNR069C (un)	*
YBL101W-A (un)	YFL002WA (un)	*	YFR047C (aa,om)	YFR047C (aa,om)	*	YLL057C (un)	YLL057C (un)	*
YBL101WA (un)	YJL162C (un)	*	YGL051W (un)	YAR033W (un)	*	YLL062C (un)	RIB4 (om)	*
YBR006W (un)	RPP2B (ps)	*	YGL096W (un)	HS449 (rp,rs)	†	YLR015W (un)	YDR469W (un)	*
YBR006W (un)	YKL023W (rt)	*	YGL161C (un)	YGL198W (un)	*	YLR046C (un)	YHL006C (un)	*
YBR052C (un)	YDR032C (un)	*	YGL198W (un)	YGL161C (un)	*	YLR065C (un)	YDL149W (un)	*
YBR077C (un)	MVP1 (vt)	*	YGL214W (un)	YLR435W (un)	*	YLR215C (un)	YLR386W (un)	*
YBR103W (ch)	YIL112W (un)	*	YGL230C (un)	KTI12 (cs)	*	YLR238W (un)	YDR200C (un)	†
YBR141C (un)	NBP35 (un)	*	YGL230C (un)	UGA4 (aa,sm)	*	YLR315W (un)	YDR383C (un)	*
YBR141C (un)	YDR372C (un)	*	YGL230C (un)	YUR161C (un)	*	YLR328W (nm)	YGR010W (un)	*
YBR190W (un)	SYF3 (rp,rs)	*	YGL242C (un)	BAS1 (aa,nm,p2)	*	YLR328W (nm)	YLR328W (nm)	*
YBR228W (un)	YLR135W (un)	*	YGR010W (un)	YGR010W (un)	*	YLR345W (cm)	MTR3 (rp)	*
YBR244W (cs)	SYF3 (rp,rs)	*	YGR010W (un)	YLR328W (nm)	*	YLR345W (cm)	SFH1 (ch)	*
YBR270C (un)	GCN3 (ps)	*	YGR017W (un)	SFP1 (cc,nc)	*	YLR368W (un)	IMP2 (pm)	†
YBR270C (un)	TAF17 (p2)	*	YGR017W (un)	YLR072W (un)	*	YLR368W (un)	THR2 (cs,om)	†
YBR270C (un)	YIL105C (un)	*	YGR024C (un)	YGR024C (un)	*	YLR368W (un)	YHR075C (en,ps)	†
YCK1 (si)	DUN1 (ri)	†	YGR058W (un)	HOG1 (cs,si)	*	YLR368W (un)	YJL048C (un)	†
YCK1 (si)	YER079W (un)	†	YGR058W (un)	YGR058W (un)	*	YLR368W (un)	YMR048W (un)	†
YCK1 (si)	YNL116W (un)	†	YGR058W (un)	YGR136W (un)	*	YLR368W (un)	YPR078C (un)	†
YCK2 (cp,si)	ADP1 (sm)	*	YGR058W (un)	YNL047C (un)	*	YLR368W (un)	YPR093C (un)	†
YCK2 (cp,si)	CTL1 (rp)	*	YGR068C (un)	CKB1 (p3)	*	YLR392C (un)	JSN1 (st)	*
YCK2 (cp,si)	GDS1 (un)	*	YGR068C (un)	SFT2 (vt)	*	YLR423C (un)	SEC35 (vt)	*
YCK2 (cp,si)	PPA2 (ph)	*	YGR122W (un)	SNF7 (cm,vt)	*	YLR424W (un)	YKR022C (un)	*
YCK2 (cp,si)	SPB1 (ch)	*	YGR154C (un)	PWP2 (cp,ck)	*	YLR432W (nm)	GCN3 (ps)	*
YCK2 (cp,si)	YER079W (un)	*	YGR173W (un)	YDR152W (un)	*	YLR432W (nm)	GDH2 (aa,om)	*
YCK2 (cp,si)	YKL204W (un)	*	YGR250C (rp)	YIR001C (un)	*	YLR432W (nm)	TAF25 (p2)	*
YCL020W (un)	YFL002WA (un)	*	YGR278W (un)	SCM4 (cc)	*	YLR432W (nm)	YDR469W (un)	*
YCL024W (cc)	NAP1 (ch)	*	YGR294W (un)	YHL018W (un)	*	YLR465C (un)	AMD1 (nm)	*
YCL046W (un)	SNF4 (si)	*,†,¶	YHL002W (un)	VPS27 (vt)	*	YML053C (un)	TDH2 (cm)	*
YCP4 (un)	YDR032C (un)	*	YHL002W (un)	YNR005C (un)	*	YML068W (un)	SNF11 (ch,p2)	*
YCR086W (me)	SCM2 (aa,sm)	*	YHL006C (un)	HDA1 (ch,pm)	*	YML088W (un)	SKP1 (aa,cc,ch,pd)	*
YDL012C (un)	CTH1 (un)	*	YHL006C (un)	YDR078C (un)	*	YML114C (un)	TAF25 (p2)	*
YDL012C (un)	GDS1 (un)	*	YHL018W (un)	YHL018W (un)	*	YML119W (un)	YLL032C (un)	*
YDL012C (un)	YFR047C (aa,om)	*	YHL046C (un)	GDS1 (un)	*	YMR025W (un)	POP2 (cm)	*
YDL012C (un)	YHR032W (sm)	*	YHR022C (un)	YIL028W (un)	†	YMR068W (un)	YIL105C (un)	*
YDL012C (un)	YHR140W (un)	*	YHR022C (un)	YNR005C (un)	†	YMR075CA (un)	YCR023C (sm)	*
YDL012C (un)	YIL172C (cm)	*	YHR039C (un)	DIG2 (di)	*	YMR077C (un)	YKL052C (un)	*



BD fusion	AD fusion	Source	BD fusion	AD fusion	Source	BD fusion	AD fusion	Source
YMR093W (un)	YDR398W (un)	*	YNL056W (un)	SIW14 (cc)	*	YNL094W (un)	SSN8 (p2)	*
YMR102C (un)	YNL218W (ds)	*	YNL056W (un)	YNL099C (un)	*	YNL094W (un)	YAL049C (un)	*
YMR210W (un)	MRP8 (ps)	*	YNL078W (un)	NAP1 (ch)	*	YNL122C (un)	YKL061W (un)	*
YMR269W (un)	GCN3 (ps)	*	YNL086W (un)	YKL061W (un)	*	YNL127W (un)	RHO4 (cp)	*
YMR312W (un)	IKI1 (un)	*	YNL091W (un)	GDS1 (un)	*	YNL127W (un)	TPD3 (cs,p3)	*
YMR322C (un)	SNZ1 (cs)	*	YNL091W (un)	YKL075C (un)	*	YNL157W (un)	ACE2 (p2)	†
YMR322C (un)	SNZ2 (cs)	*	YNL091W (un)	YNL164C (un)	*	YNL157W (un)	YJL075C (un)	†
YMR322C (un)	SNZ3 (cs)	*	YNL091W (un)	YNL288W (un)	*	YNL171C (un)	YCR106W (p2)	*
YNK1 (nm)	YNK1 (nm)	*,‡	YNL091W (un)	YPL229W (un)	*	YNL201C (cm)	AAD6 (mr)	*
YNL201C (cm)	GDS1 (un)	*	YOR220W (un)	CUP2 (cs,p2)	*	YPR105C (un)	YOR331C (un)	*
YNL201C (cm)	STD1 (cm,cs,sm)	*	YOR264W (un)	YCR086W (me)	*	YPR152C (un)	YBR194W (un)	*
YNL201C (cm)	YPR115W (un)	*	YOR264W (un)	YGR058W (un)	*	YPT1 (vt)	BOS1 (vt)	†
YNL218W (ds)	MAD2 (cc,mi)	*	YOR353C (un)	NRK1 (wm,mi)	*	YPT1 (vt)	KTR3 (pm)	†
YNL218W (ds)	YNL218W (ds)	*	YOR353C (un)	SEC35 (vt)	*	YPT1 (vt)	PEP12 (mf,vt)	†
YNL311C (un)	MET14 (aa,cs,nm,om)	*	YOR353C (un)	YOL082W (un)	*	YPT1 (vt)	YPL246C (un)	†
YNL311C (un)	YIL074C (un)	*	YPL019C (ca)	VMA22 (ca,sm)	*	YPT31 (vt)	PEP12 (mf,vt)	†
YNR004W (un)	YPL157W (un)	*	YPL110C (un)	YGR024C (un)	*	YPT31 (vt)	YKR030W (un)	†
YNR025C (un)	SEC35 (vt)	*	YPL151C (un)	PEP12 (mf,vt)	*	YPT31 (vt)	YNL146W (un)	†
YNR029C (un)	YJL064W (un)	*	YPL192C (un)	YPL192C (un)	*	YPT31 (vt)	YPL192C (un)	†
YNR029C (un)	YJL065C (un)	*	YPL222W (un)	UFD1 (pd)	*	YPT53 (vt)	SIW14 (cc)	*
YNR068C (un)	YNR069C (un)	*	YPL260W (un)	TID3 (mi)	*	YRB1 (nc)	NAP1 (ch)	*
YOL034W (un)	SPC24 (mi)	*,‡	YPR105C (un)	PEX14 (li,pt)	*	YSC84 (un)	GFD1 (un)	*
YOL070C (un)	YNL078W (un)	*	YPR105C (un)	SEC35 (vt)	*	YSC84 (un)	SLA1 (cp,st)	*
YOL106W (un)	SMX3 (ch,ds,rp,rs)	*	YPR105C (un)	TIP20 (vt)	*	YSC84 (un)	YGR268C (un)	*
YOL111C (un)	SGT2 (un)	*	YPR105C (un)	VMA22 (ca,sm)	*	YSC84 (un)	YLR243W (un)	*
YOR138C (un)	NPR2 (sm)	*	YPR105C (un)	YLR315W (un)	*	YTH1 (rp)	FIP1 (rp)	*,‡
YOR138C (un)	YGR268C (un)	*	YPR105C (un)	YMR181C (un)	*	YTH1 (rp)	KTR3 (pm)	*
YOR215C (un)	YHR115C (un)	*	YPR105C (un)	YOR164C (un)	*	ZRC1 (cs,sm)	MRP8 (ps)	*

* High throughput screen

† Protein array

‡ Previously known from two-hybrid screens

§ Previously known to interact or to occur in the same complex as shown by other methods

¶ Found in the other orientation, that is, inverted BD and AD fusions

'Cellular roles' are in parentheses; abbreviations are explained in Table 3.

Table 3 Interactions grouped by protein 'cellular roles' (as classified by the Yeast Protein Database, YPD (ref. 9) [a])

YPD classification		Results from screens			
YPD 'cellular roles'	Proteins in category [b]	Proteins identified in both screens [c]	Protein pairs [d]	Protein pairs in category [e]	
ag	Ageing	12	0	0	0
aa	Amino-acid metabolism	193	35	71	9
cm	Carbohydrate metabolism	225	38	75	8
ad	Cell adhesion	1	0	0	0
cc	Cell cycle control	136	46	114	17
cp	Cell polarity	81	20	45	4
cs	Cell stress	176	32	61	6
st	Cell structure	79	15	19	4
wm	Cell wall maintenance	152	17	25	1
ch	Chromatin/chromosome structure	174	39	81	9
ck	Cytokinesis	23	6	6	1
di	Differentiation	51	11	42	1
ri	DNA repair	106	20	34	4
ds	DNA synthesis	70	10	24	5
en	Energy generation	237	14	18	1
li	Lipid, fatty-acid and sterol metabolism	161	14	25	3
mr	Mating response	100	24	35	4
me	Meiosis	89	30	65	9
mf	Membrane fusion	23	3	18	1
mt	Mitochondrial transcription	2	1	1	0
mi	Mitosis	107	26	80	4
nc	Nuclear-cytoplasmic transport	75	26	70	9
nm	Nucleotide metabolism	83	20	35	6
ot	Other	11	2	3	0
om	Other metabolism	141	25	46	8
ph	Phosphate metabolism	20	4	14	0
p1	Pol I transcription	27	9	12	3
p2	Pol II transcription	254	65	98	20
p3	Pol III transcription	32	10	18	1
ca	Protein complex assembly	32	5	7	1
pd	Protein degradation	148	36	73	17
pf	Protein folding	53	5	9	0
pm	Protein modification	148	25	42	6
ps	Protein synthesis	338	25	46	7
pt	Protein translocation	77	10	13	3
rc	Recombination	41	13	35	4
rp	RNA processing/modification	252	49	87	32
rs	RNA splicing	102	28	58	24
rt	RNA turnover	28	7	13	0
si	Signal transduction	99	25	61	2
sm	Small molecule transport	373	31	43	3
un	Unknown	2,698	412	509	164
vt	Vesicular transport	229	52	70	15

[a] In the YPD database, proteins have been classified into at least one category, and 32% (1,109) have been placed in more than one category.

[b] Proteins in each category (categ.) based on 6,124 proteins in YPD, of which 3,426 (56%) have a cellular role assigned and 2,698 (44%) are without role assignment.

[c] Total based on the 1,004 proteins identified in the screens.

[d] Protein pairs with at least one protein in the category.

[e] Protein pairs with the two proteins in the same category.



Observation of moist convection in Jupiter's atmosphere

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The energy source driving Jupiter's active meteorology is not understood¹. There are two main candidates: a poorly understood internal heat source and sunlight. Here we report observations of an active storm system possessing both lightning and condensation of water. The storm has a vertical extent of at least 50 km and a length of about 4,000 km. Previous observations^{2,3} of lightning on Jupiter have revealed both its frequency of occurrence and its spatial distribution, but they did not permit analysis of the detailed cloud structure and its dynamics. The present observations reveal the storm (on the day side of the planet) at the same location and within just a few hours of a lightning detection (on the night side). We estimate that the total vertical transport of heat by storms like the one observed here is of the same order as the planet's internal heat source. We therefore conclude that moist convection—similar to large clusters of thunderstorm cells on the Earth—is a dominant factor in converting heat flow into kinetic energy in the jovian atmosphere.

Images were taken by the NASA Galileo spacecraft in orbit around Jupiter on 4 May 1999. The target was west of the Great Red Spot, in a cyclonic region called the South Equatorial Belt (SEB)⁴. Figure 1 of ref. 4 displays global context. The images were part of a planned effort to search for and study local convection. Earlier Galileo data showed that lightning occurs primarily at latitudes where the large-scale east–west jets produce cyclonic vorticity³. Galileo and earlier data showed that eruptions of bright cloud occur with relatively high frequency in the SEB west of the Great Red Spot^{5–7}. The successful set of images form a two-frame mosaic covering a rectangular area about 20,000 km east–west by 10,000 km north–south, about 30 degrees west of the Great Red Spot. Three sets of images were taken at different viewing angles on the illuminated portion of the planet. One set of images was taken on the dark side of the planet, after the target region reached sunset. The later sets of images on the illuminated side of the planet lag behind the first set by 0.77 and 1.55 hours. Spatial resolution (one pixel) is about 30 km. By comparison, the pressure scale height (*e*-folding height) is about 20 km near cloud level.

Figure 1 displays images showing the storm region, night-side lightning locations, and inferred wind vectors. The brightness data from the background clouds outside the storm are well fitted with a sheet of cloud located at a pressure of 0.9 bar, a haze between about 0.9 bar and 0.2 bar, and another less dense haze extending above 0.2 bar. The optical depths of the three components are about 6, 3, and 0.1 respectively. The sheet of cloud is probably ammonia ice. Its optical depth is highly variable from place to place. Most of the contrast visible in Fig. 1 outside the two storm centres is due to variability in this cloud layer. This ambient cloud structure is consistent with previous conclusions^{8,9} for most of Jupiter. Within the storm the situation is very different. Deep, optically thick cloud is required to match the data there, as described in Fig. 1. Cloud

condensation extends to at least a pressure of 3 bar, where the temperature is high enough so that nothing except water is predicted to condense¹⁰. Figure 2 presents a zoomed view of the west (left) storm and its immediate surroundings, together with a schematic cross-section illustrating cloud heights.

The night-side images do not show any features other than bright spots due to lightning. Experience with day-side imaging indicates that the *a priori* error in camera aim is ± 20 pixels (600 km or 0.5° of latitude at the current resolution). Therefore the lightning flashes are definitely associated with the two bright storm centres in the day-side images. The lightning overlay displayed in Fig. 1b is without any corrections to the predicted camera pointing. The

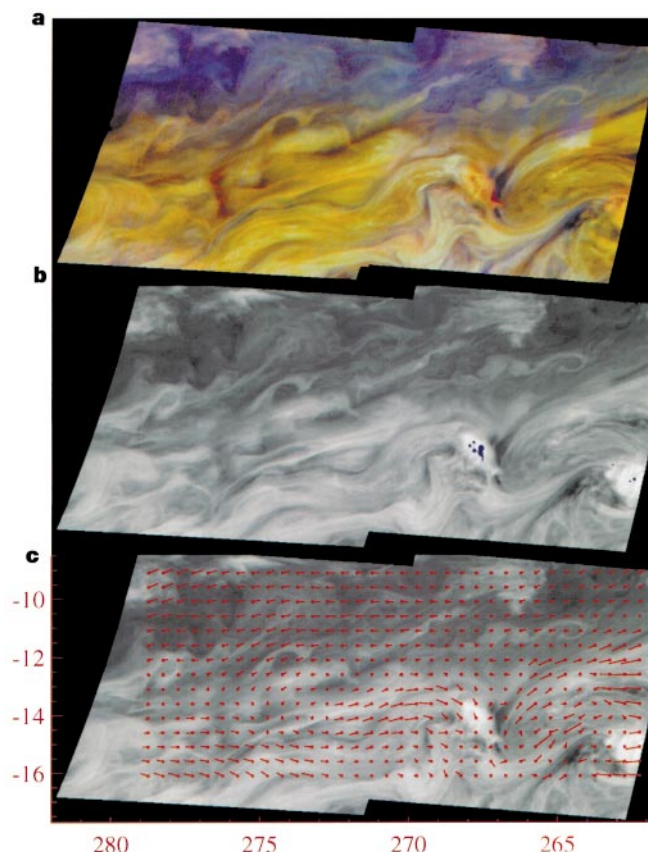


Figure 1 Mapped images of the storm region. There are two storm centres, near latitude 14 S, longitude 268 W and latitude 15 S, longitude 263 W, respectively. Detailed analysis has been carried out only for the left (west) storm. **a**, Colour images in a strong methane band (889 nm, blue), in a less strong methane band (727 nm, green), and at a continuum wavelength with negligible gas absorption (756 nm, red) are superposed. Sunlight penetrates to about the 0.5-bar level in the strong methane band and to 3 bar in the weak methane band, but the continuum penetrates more deeply. A cloud that is visible in the continuum but not in the methane band images is thus at a pressure greater than 3 bar. In practice, radiative transfer models are used to find cloud structure in local regions, following a procedure described elsewhere⁸. The strongly red feature on the southeastern corner of the storm near 268 W, 14 S is unusually dark in both methane bands and represents deep cloud. **b**, Night-side images in blue are superposed on a day-side continuum image, showing points where lightning appears in the night-side images. **c**, Map of velocity vectors. Latitudes are planetocentric. The longest vector represents 70 m s⁻¹. Markers point downwind from the grid points. A large-scale current is from west to east near the north edge of the domain, and opposite near the south edge of the domain, consistent with previous measurements of Jupiter's atmospheric jets¹¹. The region between these currents has cyclonic vorticity. Local flow near the storms is complex. The flow loop at 265 W, 14 S, just to the east of the storm, shows counterclockwise rotation, corresponding to local anticyclonic vorticity. In contrast, there is strong cyclonic shear at 269 W, 13 S, just to the northwest of the storm.

exposure time was 6.4 s, much longer than the duration of a single flash, and therefore the lightning in Fig. 1b represents clusters of flashes.

The time-lapsed images give cloud motions. Since the two storm systems in the present images are at a latitude where previous measurements of the large-scale velocity field show slow velocities¹¹, we used the cores of the storms as references. A rough estimate of position uncertainty is one image pixel, which translates into velocity uncertainty of about 5 m s^{-1} for our longest time interval. In practice, because of low contrast in much of the cloud scene, our typical uncertainties are probably about twice this. Figure 1c shows results of analysis using all three time steps. Displacements were

evaluated by shifting local regions of the time-lapsed frames and maximizing the cross-correlation. At a few locations with small-scale features and strong flow curvature, features were tracked by eye. A similar technique was used on previous Galileo images¹², but in the present case, an automated procedure dealt with a sequence of three time steps simultaneously.

Figure 1c shows a current that originates just west of the storm core and flows off to the west. Flow divergence exists near the origin of the current. We estimate that the integrated divergence over an area $1,000 \text{ km}$ by $1,000 \text{ km}$ is $20 \text{ km}^2 \text{ s}^{-1}$, with an uncertainty of at least a factor of two. In order to supply this fluid, upwelling from below is required, since the overlaying region is stably stratified and would not be expected to descend. Another feature of the local flow near the storm is the occurrence of vorticity of both signs, implying that both cyclonic and anticyclonic eddies are being generated by the storm⁴.

If we assume that all lightning storms on Jupiter are like this one, lightning storm frequency³ can give an estimate of the total heat flux carried. Previous estimates in one latitude zone⁸ suggested that moist convection might carry significant flux. The temperature excess in convective updrafts in intense storms on Jupiter has been estimated from cloud height to be about 5 K , assuming that the fluid ascends until it reaches its level of neutral buoyancy⁸. If we assume that the outflow from the storm is one scale height deep with its base at a pressure of one bar, the observed outflow from the $1,000 \text{ km}$ by $1,000 \text{ km}$ area implies a total power carried by sensible heat of about $5 \times 10^{15} \text{ W}$. The number of lightning storms discovered by Galileo imaging in late 1997 was $0.66 \times 10^{-9} \text{ km}^{-2}$ (ref. 3). Multiplying by the power per storm gives an average heat flux of 3.3 W m^{-2} . Jupiter's internal heat flux, averaged over its surface, is about 5.7 W m^{-2} . The uncertainty in the divergence rate is large, and the true depth of the outflow is also not well known. Nevertheless, it appears possible that moist convective storms carry a large portion of the heat flux through the atmospheric shell between pressures of several bars to less than one bar.

These storms appear to be analogous to clusters of thunderstorm cells on Earth called mesoscale convective complexes^{13–15}. They are typically a few hundred kilometres in diameter but can be as large as $1,000 \text{ km}$. They have lifetimes of more than 12 h and sometimes of a few days¹⁵. They tend to form where the vertical shear in the mean wind is small. The jovian cases are observed near a null in the mean zonal wind profile, which may also correspond to weak vertical shear. Mesoscale convective complexes are usually associated with cyclonic low-level winds, large-scale ascent at mid-troposphere levels, and anticyclonic high-level circulation. They are accompanied by heavy precipitation. They occur over oceans at low latitudes and over mid-latitude continental regions in the summer. The convective complexes are formed by mesoscale circulations, in contrast to squall lines, which are formed by large-scale waves with air mass contrasts. If the analogy with jovian storms is valid, it suggests that large-scale convergence exists at low levels beneath the storms, where moisture resides. □

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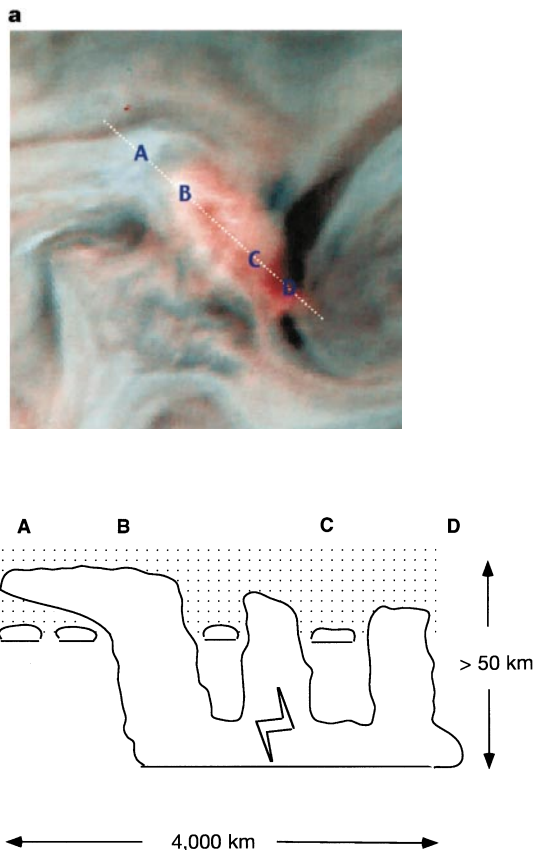


Figure 2 Expanded view of storm with colour coding to bring out deep cloud in red, and sketch of storm structure. **a**, Image: the continuum is in red, and the weak methane band is in green and blue. White regions are higher cloud, red regions are deeper cloud. **b**, Sketch: dotted regions show haze. Cloud outlines show optically thick cloud with opacity of at least 5 to 10 optical depths, caused by to condensation of water at the deepest levels and possibly a combination of water, ammonia and ammonium hydrosulphide at shallower levels¹⁰. The southeastern corner (D) is particularly dark in the methane band. It is fitted well by models that contain the two high haze layers plus an optically thick cloud at a pressure level (p) of 3 bar or greater, and no sheet cloud at 0.9 bar. This is indicated in the schematic by a small foot extending from the right hand edge of the deepest cloud. Across the central portion of the storm, high brightness in the continuum requires a deep cloud, but analysis of local brightness variations shows that there is also a spatially variable cloud near 0.9 bar. Finally, analysis of the bright spots (A) just northwest of the storm shows that optically thick cloud has penetrated up to at least the 0.5-bar pressure level. Analysis of spatial variations in brightness suggests that the deep cloud, visible in the continuum, is more spatially homogeneous than the upper clouds. This sketch is meant only to illustrate heights schematically. In particular, where thick cloud pushes to high elevations, the sketch indicates a solid tall tower of cloud, but in fact the data gives no information beneath the highest optically thick surface. In addition, cloud bases might be even deeper than indicated.

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Moist convection as an energy source for the large-scale motions in Jupiter's atmosphere

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Jupiter's dominant large-scale weather patterns (dimensions $\sim 10,000$ km) are zonal jets and long-lived ovals. The jets have been flowing east and west at constant speeds of up to 180 m s^{-1} for over 100 years^{1–3}. These jets receive energy from small-scale eddies, which pump¹ eastward momentum into the eastward jets and westward momentum into the westward jets. This momentum transfer was predicted by numerical models⁴ before it was observed on Jupiter¹. The large ovals roll between the jets in an anticyclonic direction⁵—clockwise in the northern hemisphere and counterclockwise in the southern hemisphere—where they regularly assimilate small anticyclonic eddies^{5,6}. But from where the eddies receive their energy has been an open question. Here we argue that the eddies, which ultimately drive both the jets and the ovals, receive their energy from moist convection. This hypothesis is consistent with observations of jovian lightning^{7–9}, which is an indicator of moist convection^{10,11}. It also explains the anticyclonic rotation and poleward drift of the eddies⁵, and suggests patterns of upwelling and downwelling that resemble the patterns of large-scale axisymmetric overturning in the Earth's atmosphere.

Jupiter is divided into dark and bright bands, called belts and zones, that circle the planet at constant latitude (Fig. 1). The darker belts have a disturbed, chaotic appearance; the lighter zones are more uniform. The zonal jets are located on the boundaries between the belts and zones, with the westward jets on the poleward edges of belts and the eastward jets on the poleward edges of zones. Thus the belts are cyclonic and the zones are anticyclonic.

The Galileo data^{7,9} tell us that lightning occurs mainly in the belts, and is associated with optically thick, high white cloud clusters that appear suddenly and grow to have diameters of over 1,000 km in a matter of days^{8,9}. The tops of the clusters are at pressures of a few hundred millibar, where NH_3 , H_2S , and water are possible condensates. Near the clusters there are clouds at pressures greater than 3 bar^{8,9}, where water is the only condensate. The tops of these clouds

are 50 km below the tops of the clusters. We estimate, based on the inferred abundance of water in the deep atmosphere (assuming the abundance ratios O/H, C/H, N/H and S/H are three times those in the Sun)^{12,13}, that the base of the water cloud is at ~ 6 bars. From the base to the top of the cloud clusters the vertical distance is ~ 80 km, or 0.1% of Jupiter's radius. Water is the principal agent of cloud electrification^{10,11}, as the other condensates are thought to be less abundant than water.

We argue here that the belts have a net rising motion at the cloud base, as lightning implies moist convection and moist convection requires a supply of moisture-laden air from Jupiter's interior. Other observations^{14–16}, dating back at least to Voyager, imply just the opposite—net rising in the zones and sinking in the belts—at least at the cloud-top level where the observations are most sensitive. When both views are considered together, they imply that, in the belts, the two vertical currents converge within the clouds. In the zones they diverge, according to this view. Vertical convergence must be balanced by horizontal divergence and vice versa, leading to a flow within the clouds from belts to zones.

On small scales, the situation is more complicated. The belts may resemble the Earth's Intertropical Convergence Zone (ITCZ)^{17,18}, which is the rising branch of the Hadley cell. There, the large-scale upwelling is the average of intense upwelling in small convective clusters and slower downwelling in the larger cloud-free regions¹⁷. On Jupiter, the high, thick clouds of the convective clusters exist alongside some of the clearest regions on the planet—the so-called five-micron hot spots^{3,15}. The convective clouds penetrate higher than the average cloud top, and the hot spots extend deeper than the water cloud base. This mixture of processes gives the belts their disturbed, chaotic appearance. Averaged over the entire belt, the net effect is upwelling at the base of the clouds and downwelling at the tops, according to our interpretation.

Flow from belts to zones is analogous to the Earth's Ferrel cell, which is forced by eddy heat and momentum transports^{17,18}. Here we discuss the momentum transport. On Earth the convergence of the eddy momentum flux produces a maximum eastward acceleration near the tropopause at $\sim 45^\circ$ latitude. This eastward acceleration is balanced by an inflow of high-latitude air, which has lower angular momentum and hence spins more slowly, providing westward acceleration. The flow toward the equator is analogous to the flow that we are postulating on Jupiter. There, the eddy momentum flux produces a maximum eastward acceleration at the eastward jets, which by Earth analogy should be balanced by an inflow of high-latitude air. Since the eastward jets have a belt on the poleward side and a zone on the equatorward side (Fig. 1), the flow is from belts to zones. The eddy momentum flux also produces a maximum westward acceleration at the westward jets. This is balanced by poleward flow, which is again from belts to zones.

If we assume that the eddies are moist convective structures, then we have a self-sustaining cycle: The eddies maintain the jets, and the interaction produces a pattern of upwelling and downwelling that helps maintain the eddies. There is no violation of thermodynamic principles as long as the eddies have a separate source of energy. Two-dimensional turbulent flows (vertical scale $\ll$ horizontal scale) have dynamical constraints not found in three-dimensional flows. In two dimensions, vortices grow by merging, and on a rotating planet they merge into cyclonic and anticyclonic bands^{4,19–23}. Here we argue that the small vortices get their energy from the deep atmosphere and interior through moist convection.

There are other properties of the eddies that are consistent with moist convection. The region northwest of the Great Red Spot (GRS) is the best example⁵ because the eddies there are large and easy to follow (Fig. 1). This region is part of the South Equatorial Belt (SEB), where lightning was seen in bright white cloud clusters⁹. These clusters appear suddenly and grow rapidly, and are either torn apart in the cyclonic shear of the SEB or drift poleward and become stable anticyclones moving west with the current on the southern



Figure 1 Belts and zones in Jupiter's atmosphere. The dark bands on either side of the equator are the North and South Equatorial Belts—the NEB and SEB, respectively. The southern edge of the SEB appears brighter and more disturbed than the northern edge. The SEB lies between latitude -6.0° and -17.5° , and extends to the west of the Great Red Spot (GRS) which is visible at right. The smooth bright band to the south of the SEB is a

zone, and there are several more belt-zone pairs further south. The small spots on the southern edge of the SEB are travelling west and will eventually encounter the GRS from the east. Some spots will pass around the GRS, and others will merge with it. The spots form within the SEB and emerge from it on the southern side. Lightning is associated with the spots during their formation. This is Voyager image P-20959.

edge of the SEB⁵. They encounter the GRS after circling the planet, and usually merge with it. Thus the eddies in the SEB are born as moist convective structures complete with lightning, and they die out by giving their energy either to the SEB or to the GRS.

The fact that the eddies emerge from the belts as small anticyclones⁵ is consistent with a moist convective origin. Moist convection brings mass up from below cloud base, creating a region of horizontal divergence within the clouds²⁴. On a rotating planet, divergence produces an anticyclonic structure^{17,18}. Both cyclonic and anticyclonic structures exist within the belts⁹, although the larger pattern there is cyclonic.

The fact that the anticyclones drift poleward is more puzzling, because on a rotating planet anticyclones normally drift equatorward^{25–27}. We consider the northern hemisphere, where anticyclones rotate clockwise. On the right side this rotation brings air down from higher latitudes, and on the left side it brings air up from lower latitudes. Each air parcel keeps its original amount of absolute vorticity—twice the vertical component of absolute angular velocity, which includes a part due to the planet's rotation and a part due to the parcel's rotation relative to the planet^{17,18}. Normally the planetary vorticity dominates, so the absolute vorticity increases to the north. This means that the parcels on the right have positive (counterclockwise) vorticity and those on the left have negative (clockwise) vorticity. These side vortices combine to push the main vortex southward. A different situation applies on Jupiter. There the vorticity gradients associated with the zonal jets are larger than those associated with the planet's rotation. At the latitudes of the westward jets the absolute vorticity increases to the south^{1,2}. This is the opposite of what it does on a uniformly rotating planet. It causes the side vortices to have signs opposite to those in the above example, and the anticyclones drift poleward^{126,27}.

Normally when the absolute vorticity increases to the south at some latitudes and to the north at others, the flow is unstable—it violates the barotropic stability criterion^{17,18}. This unstable property of Jupiter's zonal jets has been known since Voyager^{1,2}. In numerical

models this instability spawns waves and eddies that take energy out of the zonal jets, which become broader and weaker^{6,19–23}. When small-scale eddies are continuously forced into the flow, the jets that arise are broader and weaker than Jupiter's. In these models, the planetary vorticity dominates, and the absolute vorticity increases to the north only. Thus far it has been impossible to spontaneously generate steady zonal flows that violate the barotropic stability criterion. Our hope is that more realistic models of the eddy forcing, using moist convection as the energy source, will be more successful. □

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Structure and bandgap closure in dense hydrogen

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The possibility that steadily compressed hydrogen might undergo a transition from a proton-paired insulator to a monatomic metal was first suggested in 1935 (ref. 1). But experimental realization of metallic hydrogen in solid form has remained elusive, despite studies at pressures as high as 342 GPa (ref. 2). The pairing structure is known to be robust (from the persistence of its associated vibron mode³), leading to the suggestion of an alternative route to the metallic state, involving a band-overlap transition in which the pairing is preserved⁴. Here we report density functional calculations within the local density approximation that predict a range of densities for hydrogen where a paired or molecular metallic state may be energetically preferred. The transition to this metallic state is naturally associated with the closing of an overall bandgap; but the pressures required to effect the transition are shown to change significantly when the gaps are corrected by approximate inclusion of many-electron effects. The implication is that a complete resolution of the structural and phase problem in dense hydrogen may require methods beyond the local density approximation.

Experimentally it is known that hydrogen can exist as a low-temperature rotational crystal (phase I) to very high pressures ($P < 110$ GPa). The entry into phase II ($110 \text{ GPa} < P < 151 \text{ GPa}$) is marked by a change to wide-angle libration and hence to a continuing incoherence of motion between different molecules. However, beyond the fact that protons remain paired over a considerable density range, their time-average locations are to date experimentally unknown at high compressions. Here we extend theoretical work directed towards the determination of the optimal crystal structure with techniques which have included band-structure methods for static lattices^{4–9}, quantum Monte Carlo methods^{10,11}, and also a constant-pressure molecular dynamics simulation¹². Small energy differences between competing struc-

tures make an exact determination of the ordering difficult, but for the higher pressures (phases II and III) there now seems to be convergence toward the orthorhombic class, although the lattice parameters and physical bases are not always simultaneously optimized. Calculations⁹ (without, however, complete unit cell relaxation) showed that release of the common molecular centre of mass constraint to the ideal sites in the orthorhombic structures $Cmc2_1$ and $C2/m$ (Fig. 1) in fact led to a departure of the molecules from those sites, accompanied by an electronic charge asymmetry about the proton pairs. This effect has been proposed as a possible origin of the observed infrared vibron activity in phase III ($P > 151 \text{ GPa}$) (refs 13, 14); the implied self-consistently established dipolar character of the emerging charge distortion, and its coupling to the permanent quadrupolar components, has much to do with the form of the phase line between phases II and III^{15–17}.

As Fig. 1 indicates, the relevant orthorhombic structures appear to differ just in the location and orientation of proton pairs within the unit cell. Calculations¹² (with both unit cell relaxation and gradient corrections) predict that the structure $Pca2_1$ may be favoured, at least in phase II. With well converged results that now include complete unit cell relaxation, we find that $P2_1/c$ (not $Pca2_1$, nor $Cmc2_1$) is favoured in phase II, and that this structure also develops electronic charge asymmetry, again with a small departure of the molecules from the ideal sites. Additional studies⁹ suggested that a molecular orthorhombic structure ($Cmca$) might be preferred over (molecular) hexagonal close-packed ($mhcp-o$, or $Cmc2_1$) beyond ninefold compression. The results we present here indicate that it may be metallic and energetically favoured across a wide range of pressures beyond any of the insulating phases previously studied (and also before any metallic monatomic phases are encountered). The procedures leading to this conclusion are initially based on standard density functional theory methods executed within the local density approximation (LDA). The enthalpies of the molecular phases ($P2_1/c$, $Pca2_1$, $Cmc2_1$, and $Cmca$) and also of the monatomic semi-metal phases (β -Sn, Cs-

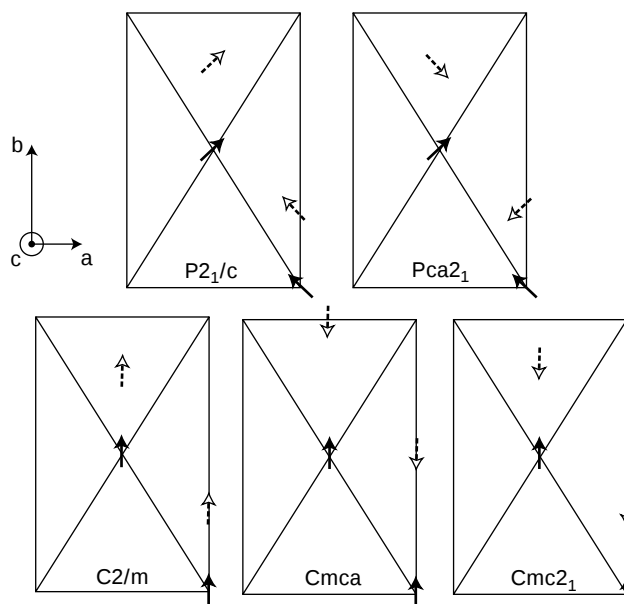


Figure 1 The principal orthorhombic structures found to be energetically competitive for static compressed hydrogen in paired form. Here the arrows indicate proton pairs, and the arrow head indicates a proton above the plane. Solid arrows signify that a pair lies in the $c = 0$ plane; dashed arrows are associated with an intermediate $c = 1/2$ plane. Of the high-pressure insulating structures $P2_1/c$ is preferred energetically, and it develops electronic charge asymmetry. Of its infrared-active modes, the principal breathing mode is least susceptible to the incoherent dephasing that accompanies the significantly anharmonic wide-angle libration⁹.

IV and diamond) are all of theoretical interest. The insulating $Pca2_1$ phase has been found⁶ to be favoured below 240 GPa with a transition to the β -Sn structure, predicting therefore an insulator–metal transition to a monatomic phase. The present results, summarized in Fig. 2, predict a $Pca2_1$ to (monatomic) β -Sn transition to occur at 265 GPa, in rather close agreement. As between $P2_1/c$ and $Pca2_1$, we find $P2_1/c$ to be favoured at all densities with a transition to β -Sn at 280 GPa (the enthalpies of the Cs-IV and β -Sn phases are nearly indistinguishable at this transition).

The $P2_1/c$ phase yields to the $Cmca$ phase at an LDA pressure of about 135 GPa. This phase is paired and since a gap has closed it is metallic; as Fig. 2 shows, it is significantly preferred over other structures until a predicted transition to the Cs-IV structure at 425 GPa. We caution that the LDA appears to underestimate the pressure in solid hydrogen compared to experimentally reported pressures^{18,19}; at $r_s = 1.51$, for example, the LDA pressure is 110 GPa while the measured value is ~ 150 GPa. (Here r_s is defined by $(4\pi/3)r_s^3 a_0^3 = N/V$, for N protons in a volume V ; the quantity a_0 is the Bohr radius.) Thus 425 GPa (LDA) will convert to a considerably higher pressure using the extrapolated experimental scale. There is negligible difference between the LDA equations of state for the insulating orthorhombic phases studied, and the

underestimate of $\sim 25\%$ is apparently quite insensitive to structure. Gradient corrections can bring the calculated value to within 20%, but some additional lowering at the level of a few per cent may also be expected from inclusion of proton dynamics. The c/a ratio at a given LDA pressure is also significantly lower than that measured¹⁸, but the use of the experimental equation of state for a given density actually brings the calculated c/a results to within 1%.

Entry into a metallic phase should be preceded by bandgap closure, and the principal gaps in the energy-bands of the orthorhombic structures $mhcp$ -c (provided for reference), $Cmc2_1$, and $P2_1/c$ structures are therefore presented in Fig. 3. Bandgap closure occurs at $r_s = 1.51$ ($mhcp$ -c), $r_s = 1.46$ ($Cmc2_1$), and $r_s = 1.38$ ($P2_1/c$) with corresponding LDA pressures of 110 GPa, 150 GPa and 220 GPa, respectively. If the experimental equation of state is again adopted, the pressures for bandgap closure shift to 150 GPa, 200 GPa and 320 GPa for $mhcp$ -c, $Cmc2_1$, and $P2_1/c$, respectively. However, the Kohn–Sham eigenvalues normally used to determine bandgaps considerably underestimate the experimental values, and an opening of the bandgap in solid hydrogen implies that an insulator–metal transition could occur at a density significantly higher than predicted in the LDA (assuming no other phase lines are crossed in the path to metallization). A solution to this familiar difficulty, reproducing the experimental bandgap of insulating systems to within 0.1 eV, is the so-called GW method²⁰, but only a limited set of GW calculations have been performed in dense hydrogen²¹ (and then only for $mhcp$ -c). Another is an approximate rendition of the same GW method found capable of reproducing bandgaps to within ~ 0.2 eV of experimental values²². It emerges from an extension of the tight-binding approach to the GW method²³ and it can be readily utilized over the large range of hydrogen densities and structures encountered here, requiring for its implementation only the charge densities and static dielectric constant. The latter can be taken from experiment at low pressures, and from extrapolation at high pressures²⁴.

An essential corroboration of the accuracy of the corrections obtained this way is to make comparison with the full GW results for $mhcp$ -c which are available from normal density to ninefold compression²¹. Figure 3 displays bandgaps of $mhcp$ -c corrected according to ref. 22. Estimating bandgap closure by linear extrapolation against density, the metallization of $mhcp$ -c is shifted from $\rho = 0.32 \text{ mol cm}^{-3}$ ($r_s = 1.51$) to $\rho = 0.39 \text{ mol cm}^{-3}$ ($r_s = 1.47$), in quite satisfactory agreement with the full GW results. Corrections by this method can be readily applied to the bandgaps of $Cmc2_1$ and $P2_1/c$, and they now indicate closure (again by linear extrapolation in Fig. 2) at densities of, respectively, $0.475 \text{ mol cm}^{-3}$ ($r_s = 1.41$) and 0.56 mol cm^{-3} ($r_s = 1.34$). The corresponding insulator–metal transition pressures obtained from use of the experimental equation of state are then 280 GPa (for $Cmc2_1$) and 410 GPa (for $P2_1/c$) at these densities. Note that the approximate linear decline of the gaps with density is a feature seen experimentally for direct gaps²⁵.

The premature entry into a metallic phase predicted by the LDA is certainly not supported by experiment, but it nevertheless can now be accounted for. Examination of the relative ordering of the insulating structures in phase II (shown in Fig. 2 inset) is, from lowest to highest, $P2_1/c$, $Pca2_1$, $Cmc2_1$ then $C2/m$ (not shown). The widest bandgaps are found to be associated with the lowest energy configurations (consistent with refs 5 and 7). At these pressures, the empirical bandgap correction is relatively insensitive to structure and does not alter the LDA conclusions. In all cases, LDA bandgap closure occurs at a density where the respective orthorhombic insulating structure relaxes into $Cmca$; this is indicative of the prominent role played by the band structure energy and, as we emphasize below, the emerging need to go beyond LDA. Bandgap corrections to the valence bands of the insulating phases must lower the total energy, suggesting that insulating phases can exist and may remain energetically favoured at pressures considerably higher than found within the LDA. Enthalpies of the metallic phases need no

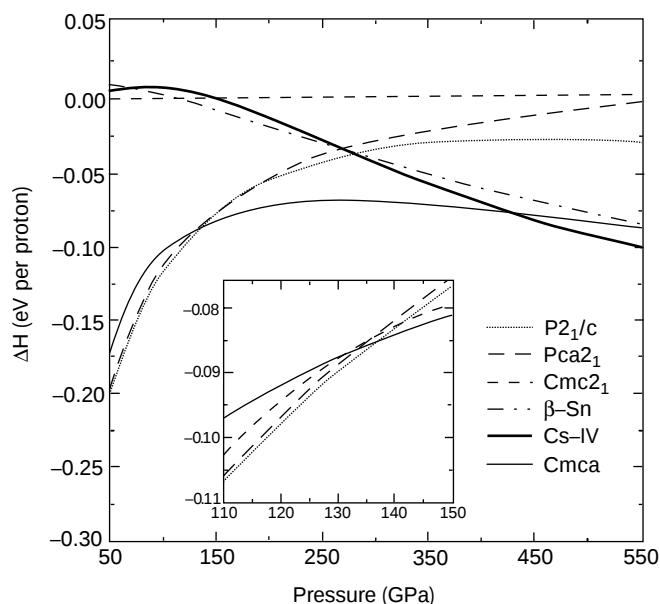


Figure 2 Enthalpy curves (relative to the monatomic diamond structure) as functions of (LDA) pressures for the molecular and monatomic structures studied. Thin lines represent monatomic phases, thick lines represent molecular phases. Inset, details of the low (LDA) pressure region associated with phase II of solid hydrogen. (The difference in order from the results of ref. 12 may result from the use of a non-plane-wave basis-set determined from wavefunctions at the Γ -point.) These results are from *ab initio* calculations²⁸ performed for lattices of static protons to determine optimal unit cell structural parameters (c/a and b/a ratios) and fully relaxed proton coordinates. Gradient corrections are found not to alter the relative energies. An ultrasoft pseudopotential and a bare $1/r$ potential have both been used for the proton–electron interaction²⁹; structural results for the two consistently agreed to within 0.5%. The energy cut-offs were 450 eV and 2,040 eV respectively for the corresponding planewave basis sets, and are adequate to converge the total energy to within 10 meV per proton for the former and 20 meV for the latter. Integration of the charge density is carried out on a $20 \times 12 \times 12$ $\mathbf{k}$ -point mesh for the molecular phases, which is evidently sufficient to converge the total energy to 0.5 meV per proton. Relaxation of proton coordinates continues until forces are reduced to below 1 meV Å^{-1} , with energies converged to better than 1 meV per proton; equivalent accuracy is also achievable with respect to $\mathbf{k}$ -point selection for monatomic phases. Structural features are estimated to be converged to better than 0.5%, and the relative energy difference between structures to within 2 meV per proton (ref. 30). As is evident, the $Cmca$ structure is strongly preferred over a wide range of pressures.

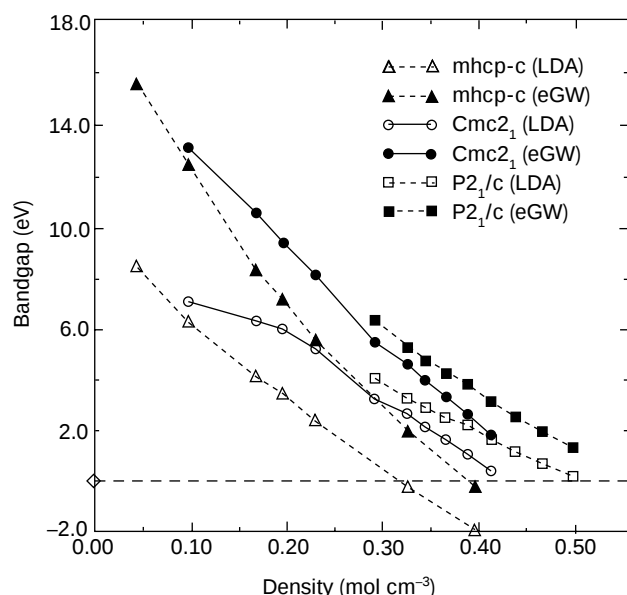


Figure 3 Examples of bandgaps as a function of density for the *mhcp-c*, *Cmca2₁*, and *P2₁/c* structures of dense hydrogen. Open symbols indicate LDA bandgaps, and closed symbols represent empirically corrected bandgaps (eGW). Results for *C2/m* and *Pca2₁* are relatively close to the corresponding values for *Cmca2₁* and *P2₁/c*, the bandgaps being smaller in each case. Typical corrections are 1.5–2.0 eV at the higher densities; the highest occupied valence-band state at the zone centre is required for the gap corrections²². Estimates of the onset pressures of bandgap closure may be modified slightly by the tendency of the LDA to lengthen the dimer separation (to around 1.45 a_0). Use of a dimer length of 1.4 a_0 can lead to shifts of around 0.3–0.5 eV.

such energy correction, and the LDA should be sufficiently accurate to the extent that we expect little change in the relative ordering of the metallic phases. However, the enthalpy of the insulating phases is expected to be lowered with respect to the metallic phases, and in fact, the results now imply that the insulating phases may be stable up to the point of bandgap closure (below about 410 GPa for *P2₁/c* before a phase transition to *Cmca*).

We now address the role of proton dynamics, present in all phases. Coherent zero point motion (ZPM) is generally expected to reduce the bandgap by introducing factors of the Debye–Waller type into the periodic potential of the crystal. Wide-angle libration, if viewed as incoherent or uncorrelated motion (recall that a complete rotational state in $J = 0$ *para*-hydrogen corresponds to uncorrelated orientational motion at different sites), appears to enhance the bandgap⁴ although the magnitude must depend on the choice of initial ordered structure. From the data presented here, *mhcp-c*, which has usually been taken as the corresponding ordered structure in disorder studies, has the smallest bandgap at any density. Incoherent ZPM can then increase the bandgap by introducing an effective potential which averages over structures with allowed molecular orientations, some of which include the wide-gap structures *Pca2₁*, *P2₁/c* and so on. In the case of *P2₁/c* it appears unlikely that incoherent ZPM can open the bandgap further and in fact, an incoherent average of the possible states should lead to a bandgap closure earlier than indicated by the static calculations, assuming that no orthorhombic structures exist with a bandgap larger than that observed in *P2₁/c*. Caution is again necessary because (as noted) LDA appears to underestimate pressures and because differences in enthalpy between static phases are only 10–20 meV per proton; small differences in ZPE may still well be the deciding factor for the exact transition pressures. To take one example, our static LDA results favour the β -Sn structure over the

diamond structure above 110 GPa, whereas the latter structure has been predicted¹¹ to be stable up to 350 GPa when ZPE is added. ZPE has been found to tend to favour the more isotropic structures²⁶ (for example, diamond over β -Sn above). The orthorhombic structure *Cmca*, in which the molecules shift into planes separated by a distance $b/2$, is quite anisotropic compared with the insulating orthorhombic structures (where the molecules lie near to the ideal sites). The relative difference is then expected to increase the transition pressure from that given by the static LDA results.

Our results suggest that the paired state of hydrogen is tenacious at high pressures, and that a metallic pairing state may precede eventual pair dissociation. For insulating phases, *P2₁/c* appears to be the preferred structure and it possesses an electronic charge asymmetry⁸; on metallization, *Cmca* is then indicated to be the preferred structure. Corrections to LDA are, however, of demonstrated significance in bandgap closure, and an important conclusion is that further analysis of the hydrogen structural problem must proceed beyond the static LDA regime with enthalpies (and not merely bandgaps) being determined in a fully self-consistent manner. But if gap closure can be taken as the criterion, then the corrected gaps suggest that (to within possible dynamic corrections) an insulator–metal transition (and subsequent onset of Drude reflectivity) may occur in the vicinity of $p \sim 410$ GPa ($r_s = 1.34$). Finally, the possibility of a paired band-overlap state is of some experimental interest because, as has been pointed out recently²⁷, the paired metallic phase (whose electron–hole character can lead to a weakening of the Coulomb pseudopotential) presents the possibility of superconductivity in paired metallic hydrogen with significant transition temperatures. □

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Direct measurement of electrical transport through DNA molecules

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Attempts to infer DNA electron transfer from fluorescence quenching measurements^{1–9} on DNA strands doped with donor and acceptor molecules have spurred intense debate^{10,11} over the question of whether or not this important biomolecule is able to conduct electrical charges. More recently, first electrical transport measurements on micrometre-long DNA ‘ropes’¹², and also on large numbers of DNA molecules in films¹³, have indicated that DNA behaves as a good linear conductor. Here we present measurements of electrical transport through individual 10.4-nm-long, double-stranded poly(G)-poly(C) DNA molecules connected to two metal nanoelectrodes, that indicate, by contrast, large-bandgap semiconducting behaviour. We obtain nonlinear current–voltage curves that exhibit a voltage gap at low applied bias. This is observed in air as well as in vacuum down to cryogenic temperatures. The voltage dependence of the differential conductance exhibits a peak structure, which is suggestive of the charge carrier transport being mediated by the molecular energy bands of DNA.

The common model for electron transfer through DNA is based on overlap between π orbitals in adjacent base pairs¹⁴. Irregular base-pair sequences may lead to localization of charge carriers and reduce the transfer rate of electrons^{9,15}. A structure containing a single type of base pair may therefore furnish the best conditions for π overlap. In our experiments, we used 10.4-nm-long (30 base pairs) poly(G)-poly(C) DNA oligomers, well characterized by ultraviolet measurements, mass spectrometry and gel electrophoresis. The 10.4-nm size is adequate to span two closely spaced metal nanoelectrodes as a linear, nearly stiff structure. A DNA molecule was positioned between the nanoelectrodes by electrostatic trapping (see Fig. 1 legend for details) from a dilute aqueous buffer containing about one molecule per (100 nm)³. This technique was developed for the trapping of single molecules, and has

been shown to be successful for a variety of nanoparticles^{16,17}. After a DNA molecule was trapped from the solution, the device was dried in a flow of nitrogen and electrical transport was measured. No current was measured between the bare electrodes before trapping the DNA, indicating that no short-circuit, breakdown or field emission occurs in the voltage range investigated (as checked up to 5 V for every sample and occasionally up to 10 V).

Here we report current–voltage (I – V) measurements on DNA oligomers. The current is essentially zero (<1 pA) up to a threshold voltage of a few volts. This shows that this system behaves as an insulator at low bias. Beyond the threshold the current rises sharply, showing that DNA can transport charge carriers. Relatively large currents can be supported by the DNA molecule: we occasionally put more than 100 nA through our device, which corresponds to a high rate, $\sim 10^{12}$ electrons s^{-1} , over the 10-nm-long molecule. Note, however, that our experiments involve electron transport rather than electron transfer (the latter describes a one-step tunnelling process). Subsequent current–voltage measurements under the same conditions yield similar characteristics, but with a variation of the width of the voltage gap (Fig. 1). Similar results were obtained for more than 20 different samples at ambient conditions.

A number of control experiments were performed. To verify that we indeed trap DNA and no other (in)organic particles, we

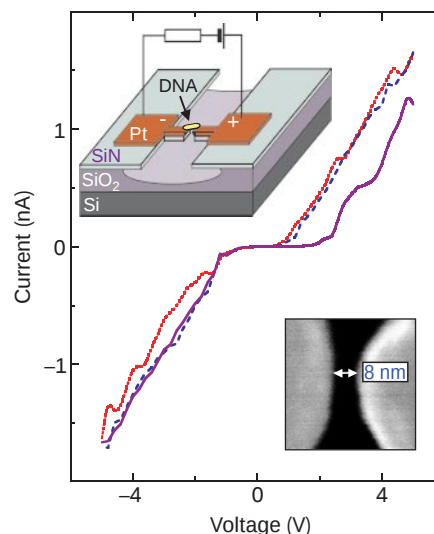


Figure 1 Current–voltage curves measured at room temperature on a DNA molecule trapped between two metal nanoelectrodes. The DNA molecule (30 base pairs, double-stranded poly(G)-poly(C)) is 10.4 nm long, and the nanoelectrodes are separated by 8 nm. Subsequent I – V curves (different colours) show similar behaviour but with a variation of the width of the voltage gap. Note that the conductance in these measurements is limited by a 2 G Ω series resistor. Air humidity is 50%. The upper inset shows a schematic of our sample layout. Using electron-beam lithography, we create a local 30-nm narrow segment in a slit in the SiN layer. Underetching the SiO₂ layer leads to two opposite free-standing SiN ‘fingers’ that become the metallic nanoelectrodes after sputtering Pt through a Si mask. The lower inset is a scanning-electron-microscope image of the two metal electrodes (light area) and the 8-nm gap between them (dark area). Details of fabrication and trapping are given elsewhere^{16,17}. DNA was prepared in a buffer composition of 300 mM NaCl, 10 mM Na citrate and 5 mM EDTA (Eurogentec Bel S.A.). Deposition of a DNA molecule between the electrodes was achieved with electrostatic trapping^{16,17}. A 1- μ l droplet of dilute DNA solution is positioned on top of the sample, and a voltage of up to 5 V is then applied between the electrodes. The electrostatic field polarizes a nearby molecule, which is then attracted to the gap between the electrodes owing to the field gradient. When a DNA molecule is trapped and current starts to flow through it, a large part of the voltage drops across the series resistor, which reduces the field between the electrodes and prevents other molecules from being trapped^{16,17}. Trapping of DNA molecules using this method is almost always successful.

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incubated a sample with trapped DNA (Fig. 2, solid curve) in a solution containing DNase I, an enzyme that specifically cuts double-stranded DNA. After the DNase I treatment, no significant current was observed (Fig. 2, dashed curve), indicating that the electrodes were indeed originally connected by DNA. In a complementary experiment, we repeated the same procedure using a similar enzyme solution but without the Mg ions that are needed to activate the enzyme. In this experiment the general shape of the curve did not change (Fig. 2, inset), confirming that in the original control experiment the DNA was indeed cut by the DNase I enzyme.

The length selectivity of the trapping procedure was checked by attempting to trap 10.4-nm-long chains between electrodes separated by 12 nm instead of 8 nm. Lack of success indicates that the DNA is trapped with its long axis bridging the two electrodes, and that it is very unlikely that we have measured conductance through two or more molecules in series, or through a number of parallel DNA chains 'sandwiched' between the electrodes, where each chain has its axis perpendicular to the line connecting the electrodes. Scanning-electron-microscope imaging of the gap between the electrodes after trapping a DNA molecule showed a very small amount of material (compared to the gap size) between the electrodes, probably amorphous carbon covering the DNA during the imaging process. Imaging of the bare electrodes before trapping the DNA did not show this material. The very low concentration of molecules in the solution, the trapping procedure (using a 2 G Ω series resistor), and the imaging together suggest that only a single DNA molecule, or at most a few, becomes trapped.

We also tried to trap from a droplet of buffer solution without DNA. Nothing was trapped in this case, and no conduction by ions or molecules present in the buffer solution was observed. I - V measurements performed in vacuum ($\sim 10^{-6}$ mbar) and below -20°C exclude the possibility of ionic conduction through traces of water around the molecule¹⁸, and confirm that the current is flowing through the DNA.

Three samples were measured in vacuum and at low temperatures down to 4 K. The differential conductance dI/dV versus V (Fig. 3a)

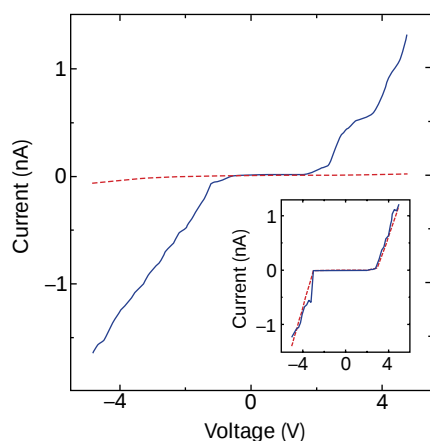


Figure 2 Current-voltage curves that show that transport is indeed measured on DNA trapped between the electrodes. The solid curve is measured after trapping a DNA molecule as in Fig. 1. The dashed curve is measured after incubation of the same sample for 1 h in a solution with 10 mg ml⁻¹ DNase I enzyme (5 mM Tris-HCl, 5 mM MgCl₂, 10 mg ml⁻¹ DNase I (pH 7.5)). The suppression of the current indicates that the double-stranded DNA was cut by the enzyme. This experiment was performed for four different samples (including the sample of Fig. 3a). Inset, two curves measured in a complementary experiment where the above procedure was repeated but in the absence of the Mg²⁺ ions that activate the enzyme and in the presence of 10 mM EDTA (ethylenediamine tetraacetic acid) that complexes any residual Mg ions. In this case, the shape of the curve did not change. This verifies that the DNA was indeed cut by the enzyme in the original control experiment.

exhibits a peak structure, with a peak spacing of 0.1–0.5 eV and a peak width of ~ 0.2 eV (typical values from many such curves). This structure is stable and reproducible over time, as can be seen from the close similarity of the six subsequent curves plotted in Fig. 3a. But after a number of I - V measurements (inset, blue line) we observe an abrupt change in the shape of the I - V curves: the new, stable and reproducible shape is shown as a pink line in the inset. Changes between stable configurations can be induced by an abrupt switch of the applied voltage or by a high current. Measurements at higher bias voltages (beyond the scale of Fig. 3a) also manifest a peak structure, but with more fluctuations and less reproducibility. The voltage gap in the I - V curves widens on increasing the temperature, as shown in Fig. 4. We do not yet understand this intriguing trend, which was observed for three different samples in both cooling and heating runs. The thermal expansion of the gap between the electrodes is very small (of the order of 1 Å), but may conceivably have some effect.

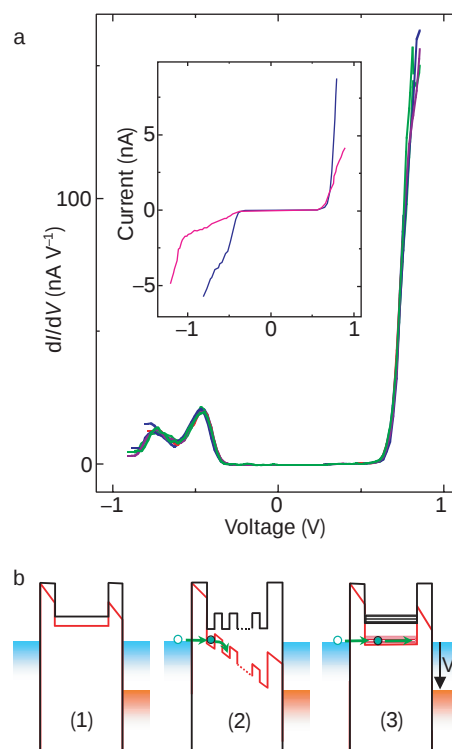


Figure 3 Differential conductance dI/dV versus applied voltage V at 100 K (**a**), and various models for the transport (**b**). **a**, The differential conductance shows a clear peak structure. Good reproducibility can be seen from the six nearly overlapping curves. The peak structure suggests that electron transport is mediated by the DNA molecular energy bands. Peak structures were observed in all three samples measured at low temperatures, although details were different from sample to sample. The dI/dV data were obtained by numerical derivation of the I - V curves. Subsequent sets of I - V measurements can show a sudden change, possibly due to conformational changes of the DNA. Inset, an example of two typical I - V curves that were measured before and after such an abrupt change. Switching between stable and reproducible shapes can occur after an abrupt switch of the voltage or by high current. The I - V curves were measured without a series resistor. **b**, Three basic models for transport through DNA. The black and red lines illustrate the situation with and without an applied bias voltage V , respectively. The contact between the DNA and the metal electrodes is represented by tunnelling barriers. The middle part describes the electronic states of the DNA. Model 1 describes unistep tunnelling, as commonly discussed in electron-transfer studies, model 2 describes sequential hopping between localized states, and model 3 describes molecular band conduction.

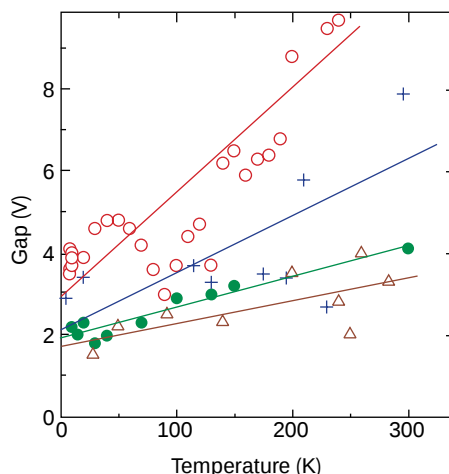


Figure 4 Temperature dependence of the voltage gap in the I - V curves for three different samples. The top two data sets (open circles and crosses) show results from two samples; the lower two data sets are cooling and heating measurements on a third sample. Solid

lines are linear fits. The voltage gap is defined by the voltage at which the current rises above 1 pA. The voltage gap appears to widen with increasing temperature.

We now consider how these data relate to available models for electron transport through DNA. Figure 3b schematically outlines three models. The first is a one-step electron transfer process that involves tunnelling from electrode to electrode^{8,9}. This can be ruled out in our samples owing to the very large tunnelling distance that would be involved (8 nm) and the large currents observed. The second model describes sequential hopping between localized states^{7,8}, which could, for example, be associated with the base pairs. The hopping process could be either unidirectional or involve one-dimensional diffusion. It can be argued that the back-and-forth diffusive hopping⁸ is less likely in our case due to the high electric fields used, which will tilt the potential, and the high electron-transfer rates observed. (A calculation of the time needed for such diffusive motion along the chain limits the electron transfer rate to a value well below the one we observe.) In the third model, electronic interactions between the bases in the DNA molecule lead to a molecular band where the electronic states are delocalized over the entire length of the molecule. Electron transport in the hopping and band models is facilitated when the Fermi level of the electrode is aligned with the band edge by applying the bias voltage. Once electrons are injected, transport occurs through hopping or band conduction. (Holes rather than electrons may be the relevant carriers, but the physical picture of the transport process is essentially the same.)

The nature of the contact resistance between the DNA and the metal electrodes is not known. However, it is very likely that there is no good metallic contact¹⁹ and that the contacts can be represented by tunnelling barriers. If the applied voltage drops largely across these barriers, the differential conductance dI/dV is determined by a combination of the electronic density of states of the DNA molecule and Coulomb blockade effects²⁰. These effects are observed when the capacitance C of an object is so small that the addition of a single electron costs a charging energy $e^2/2C$ that is larger than the thermal energy. Coulomb blockade may lead to a voltage gap and steps in the I - V curve. For a 10-nm-long DNA molecule with states delocalized over the whole molecule, we estimate $C \sim 1$ aF and $e^2/2C \approx 0.2$ V, which is one order of magnitude smaller than the voltage gap observed in our measurements. Neither do we observe clear periodic Coulomb steps in the I - V curves. Although Coulomb blockade is thus likely to make a minor contribution to the gap in this case, it cannot solely explain it.

In the simplest hopping model, the DNA may alternatively be

considered as a series of 30 very tiny (<3 Å) quantum dots. Each of these dots has a large charging energy (>5 V), and their series addition would lead to an even larger overall charging energy. This would lead to a Coulomb blockade voltage gap that is again incompatible with the data. Larger quantum dots formed from assemblies of adjacent base pairs may be considered, but this is not likely for our homogeneous poly(G)-poly(C) DNA where an extended model is more reasonable. We thus conclude that most of the observed gap probably originates from the offset between the Fermi level of the electrode and the molecular energy bands of the DNA molecule. The peaks in our curves then reflect the structure of the molecular bands of the DNA^{21,22}. □

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Non-destructive determination of local strain with 100-nanometre spatial resolution

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Structure sizes of ~ 180 nm are now standard in microelectronics, and state-of-the-art fabrication techniques can reduce these to just a few tens of nanometres (ref. 1). But at these length scales, the strain induced at interfaces can locally distort the crystal lattice, which may in turn affect device performance in an unpredictable way. A means of non-destructively characterizing such strain fields with high spatial resolution and sensitivity is therefore highly desirable. One approach is to use Raman spectroscopy², but this is limited by the intrinsic ~ 0.5 - μm resolution limit of visible light probes. Techniques based on electron-beam diffraction can

achieve the desired nanometre-scale resolution. But either they require complex sample preparation procedures³ (which may alter the original strain field) or they are sensitive to distortional (but not dilational) strain within only the top few tens of nanometres of the sample surface^{4,5}. X-rays, on the other hand, have a much greater penetration depth, but have not hitherto achieved strain analysis with sub-micrometre resolution⁶. Here we describe a magnifying diffraction imaging procedure for X-rays which achieves a spatial resolution of 100 nm in one dimension and a sensitivity of 10^{-4} for relative lattice variations. We demonstrate the suitability of this procedure for strain analysis by measuring the strain depth profiles beneath oxidized lines on silicon crystals.

The crucial element of our set-up is a waveguide^{7,8} (WG in Fig. 1), which constitutes an unusual optical element for medium- and high-energy X-rays (10–30 keV). Unlike diffracting optical elements it does not focus a collimated incident beam, but instead confines it (through a resonance effect⁹) in one direction to a dimension of typically 100–150 nm, the distance between the parallel interfaces of the resonator⁸. Several resonator modes can be excited; these modes can leave at the end of the waveguide, with a high degree of spatial coherence in the plane perpendicular to the waveguide surface, which is here the vertical one. In this direction, the beam profile of the first resonance mode—at distances of more than 1 mm from the waveguide end—is well described by a gaussian beam^{10,11}; this mode then seems to originate from a virtual line source inside the resonator. This has allowed us to register an in-line hologram with a spatial resolution of 140 nm in one direction¹².

The idea behind the present diffraction imaging experiment is as follows: for highly monochromatic X-rays, a perfect crystal has a limited angular acceptance (typically a few tens of microradians) in its diffraction plane, which in our geometry in Fig. 1 is the horizontal plane. But in the vertical plane, its angular acceptance is larger and can even exceed 1 mrad. This acceptance can be matched favourably with the characteristics of the waveguided beam, which is divergent (typically 1 mrad) in the vertical plane while maintaining its collimation in the horizontal plane. Strain sensitivity is then achieved with the collimated beam in the horizontal plane. High spatial resolution is utilized in the vertical direction, in which local structural variations at the sample are registered by projecting them, magnified, on a two-dimensional detector placed far from the sample. So spatial resolution and strain resolution are not correlated, as they are obtained in orthogonal directions: spatial resolution in the horizontal plane has to be sacrificed in order to obtain good strain sensitivity.

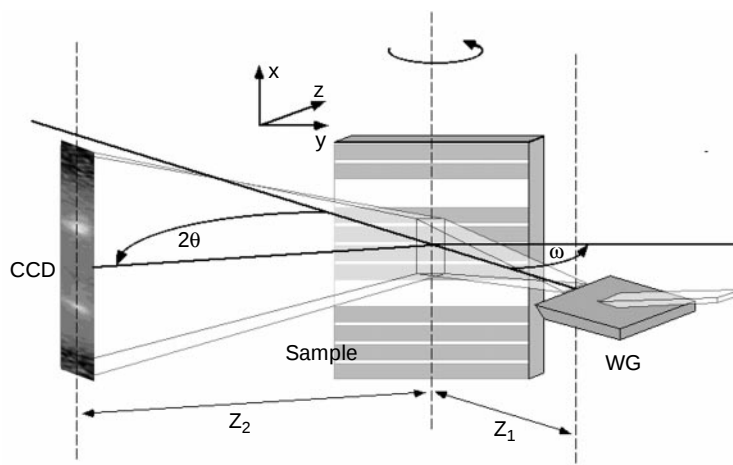


Figure 1 Diffraction imaging set-up. The beam arriving on the waveguide (WG) surface is spatially compressed by the waveguide in the vertical direction; the beam leaving the waveguide is vertically divergent. This latter beam, impinging on the sample surface at an incident angle ω , is diffracted in the horizontal plane towards the two-dimensional CCD

detector positioned at a fixed scattering angle 2θ . The waveguide disperses the beam in the vertical plane providing at the CCD detector a magnification $M = (Z_1 + Z_2)/Z_1 \sim Z_2/Z_1$ for any structural variations occurring at the sample surface in this plane. Z_1 is the WG–sample distance, Z_2 is the sample–detector distance. In a Bragg scan only ω is varied.

We used the experimental set-up in Fig. 1, with collimated and monochromatic synchrotron radiation, at the X-ray undulator beamline ID13 (Microfocus) at the European Synchrotron Radiation Facility (ESRF). The purpose of our study was an industrially relevant problem: the determination of the strain field induced in a silicon substrate by the edges of silicon oxide overlayer stripes produced by the LOCOS (local oxidation of silicon) process¹³. A pattern with several blocks of oxide stripes was prepared on a highly planar (001) Si wafer. Each block consisted of three field oxide stripes (of micrometre or sub-micrometre width), oriented along the (110) direction and separated by active zones (Si spacers) that were $\sim 4 \mu\text{m}$ wide. Wider oxide stripes (widths 5.5–7.5 μm) divided the single blocks. Figure 2 shows a height profile obtained by atomic force microscopy (AFM) at the investigated portion of the pattern: namely, the wide separation oxide, the Si spacers and two narrow oxide stripes. All oxide stripes have rounded edges due to the 'bird's beak' effect, caused by oxygen diffusion under the protective nitride mask². Here we will use the orthogonal crystal coordinates shown in Figs 1 and 2: x is perpendicular to, and y is parallel to, the stripes in the sample surface, while z is perpendicular to the surface.

A collimated X-ray beam (beam divergence $< 5 \mu\text{rad}$) – monochromatized by a double-crystal Si(111) monochromator to 13-keV photon energy (wavelength, 0.095 nm) and limited in size by a 50- μm pinhole at 30 m from the source – was compressed in the vertical direction in the waveguide. The rotation axis for the sample was aligned perpendicular to the waveguide surface, and the stripes of the patterned sample were aligned parallel to the diffraction plane. The diffracted intensity was recorded in a scan around the Si (400) Bragg reflection (angle of grazing incidence $\omega \approx 20.57^\circ$), with 8.73 μrad (0.0005°) step size, by use of a two-dimensional CCD detector (low-noise FRELON camera¹⁴) with an acquisition time of 60 s per image. As the chosen magnification, $M \approx Z_2/Z_1$, was ~ 90 ($Z_1 = 6.3 \text{ mm}$, $Z_2 = 573 \text{ mm}$; see Fig. 1 legend), and the pixel size was 10 μm , each pixel row received structural information always from the same region of the sample, extending only 110 nm along x . The footprint size (full-width at half-maximum, FWHM) in this (vertical) direction was about 5 μm , while about 145 μm (=beam size/ $\sin(\omega)$) were illuminated homogeneously along y . The spatial resolution in the y direction was therefore 145 μm , which is sufficient for the analysis of our 200- μm -long lines. We note that a better resolution of 30 μm is feasible in this direction, as a reduction in beam size to 10 μm will not deteriorate the beam collimation due to diffraction enough to affect our experiment.

The present full-field projection set-up allows the immediate detection of small local variations in the lattice parameters as

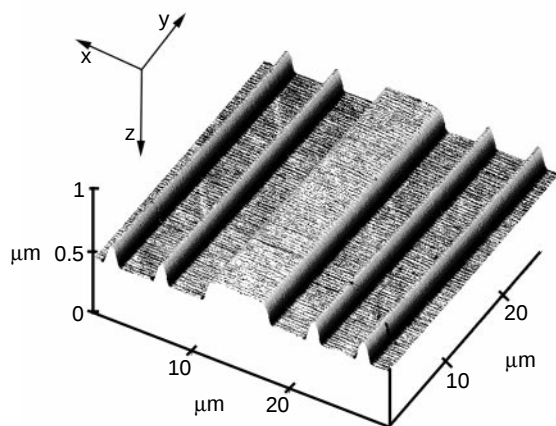


Figure 2 Atomic force microscopy scan of sample height profile. The SiO₂ stripes of different width extend $\sim 170 \text{ nm}$ above the sample surface.

intensity variations in the single images. For a more detailed quantitative analysis, the measured intensity was transferred into reciprocal space (S_x, S_z) with the relations¹⁵:

$$S_x = (-\cos\omega + \cos(2\theta - \omega))/\lambda = 2\sin\theta\sin(\omega - \theta)/\lambda \quad (1)$$

$$S_z = (\sin\omega + \sin(2\theta - \omega))/\lambda = 2\sin\theta\cos(\omega - \theta)/\lambda \quad (2)$$

where 2θ is the scattering angle as registered by the CCD detector. In the resulting reciprocal space maps (RSMs), both the variation of the crystal lattice spacing and the presence of crystal bending or mosaic structure could be detected. It is beyond the scope of this Letter to make a detailed analysis of the structural properties of this particular sample: we shall limit our discussion to the strain–depth profile. Evidence of such a profile is given in an RSM by a broadening of the intensity peak along S_z ; broadening in S_x is associated with crystal plane bending or mosaicity, which were found to be negligible in our sample. Thus we integrated the RSMs in S_x in order to obtain diffraction profiles as a function of $S_z = 1/d$, where d is the interplanar spacing of the diffracting planes under consideration. The data points in Fig. 3b show three experimental curves obtained at three lateral positions x underneath the central part of a 1- μm -wide SiO₂ stripe (here $\Delta S_z = S_z - S_{z0}$, where S_{z0} refers to the position in reciprocal space of the corresponding peak of an unstrained Si crystal, $S_{z0} = 1/d_{(400)} = 7.365 \text{ nm}^{-1}$; $d_{(400)}$ is the interplanar distance of the Si (400) planes). To interpret these results we carried out calculations of the strain profile underlying the SiO₂ stripes using Hu's analytical model^{16,17} for edge-induced stress and the principle of superposition. The standard values for the silicon stiffness constants were used¹⁸.

Figure 3a shows the calculated perpendicular component $\epsilon_{zz}(x, z)$ of the strain profile along z for the same three positions of the

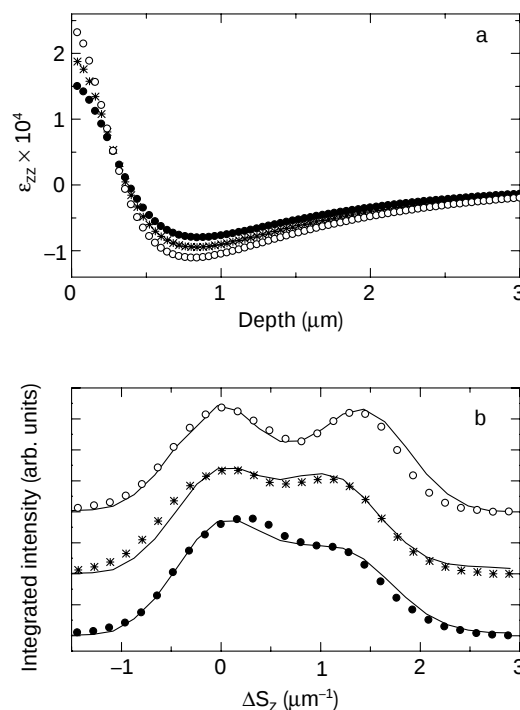


Figure 3 Strain–depth profile (**a**) and the resulting diffraction profile (**b**). **a**, Strain–depth profile calculated for several lateral distances x under a 1- μm -wide SiO₂ stripe. Open circles, data for the stripe centre position; stars and filled circles, positions 220 nm and 330 nm off-centre, respectively. **b**, Measured (plotting symbols) and calculated (lines) diffraction profiles for the same lateral positions as in **a**. (See text for definition of ΔS_z .) The calculated diffraction profiles are obtained for the strain–depth profiles of **a** and are convoluted with the instrumental resolution function that is derived from the measured diffraction profile of the perfect Si crystal.

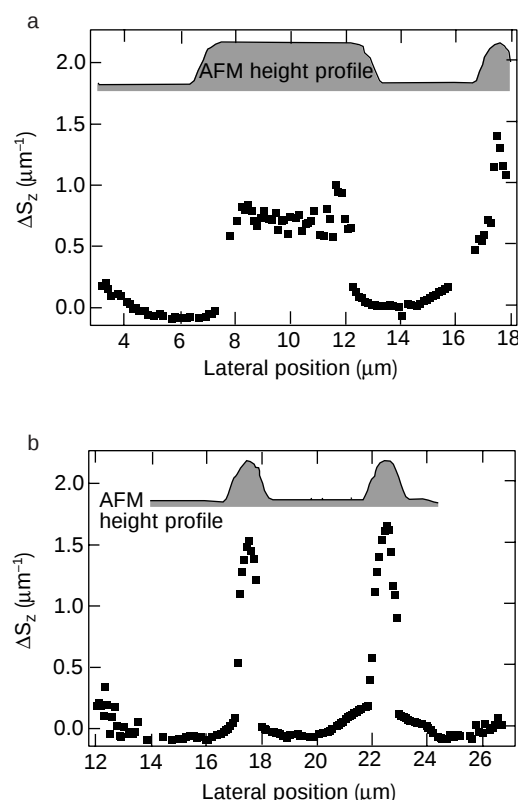


Figure 4 Spatial variation of strain under different oxidized stripes. The experimental data (symbols) indicate the position of the Si (004) Bragg peak in reciprocal space as a function of the lateral position on the sample. The data are presented as $\Delta S_z = S_z - S_{z0}$, where S_{z0} is defined in the text, and S_z refers either to the position of a fitted single peak or to the position of a detectable second peak. These peak positions are derived from fits of gaussian peaks (single or double) to the integrated $I(S_z)$ curves. The values of ΔS_z are presented for two zones of the sample corresponding to the wide SiO₂ stripe (a) and to two narrow sub-micrometre SiO₂ stripes (b). For comparison, the height profile of the sample as obtained by the scanning AFM measurements is shown as an inset at the top of each panel.

experimental curves shown in Fig. 3b, taking into account the real thickness profile of the SiO₂ stripe, obtained from the AFM measurements. These computed strain profiles, which extend for several micrometres below the surface, were used as input to simulate—by use of the dynamical X-ray diffraction theory formalism of Takagi-Taupin for distorted crystals¹⁹—the diffraction spectra shown as continuous lines in Fig. 3b. These computed diffraction profiles were convoluted with the instrumental resolution function, which can be derived from the profile measured for a perfect Si reference crystal. These simulations consistently describe the experimental data across the narrow stripe. In order to get a complete strain-profile picture of the sample, this procedure should be repeated for all the lateral coordinates x . However, our aim is to demonstrate that it is possible to follow the local strain variations along x using a simplified approach.

From the data in Fig. 3b, we find that the experimental diffraction profiles registered in a strained region can be fitted with two gaussian-shaped peaks. In contrast, the spectra from other parts of the sample—where the strain field vanishes—can be fitted with only one gaussian peak. Applying a gaussian peak (single or double) fitting procedure to all experimental data (that is, to all $I(S_z)$ diffraction profiles obtained for all lateral positions x on the sample surface), either the position S_{z1} of the single peak or the position S_{z2} of a detectable second peak were obtained for all lateral

positions x (plotted in Fig. 4). Figure 4a shows results for a zone with a wide SiO₂ stripe, and Figure 4b the results for a zone with two narrow SiO₂ stripes. Even though our approach is rather empirical, if we consider the values of ΔS_z as a measure of the internal strain, the observed features are in agreement with the calculations: the strain has the absolute maximum under the central part of the narrow sub-micrometre stripes. Under wider stripes we find relatively smaller strain with local maxima, as expected from theory^{16,17}, at the edges of the oxidized stripes just where the 'bird's beak' deformation sets in.

We now consider the lateral resolution of our technique, and its sensitivity. The limitation on the required deconvolution of a second peak along S_z , caused mainly by a modest energy resolution ($\Delta E/E=10^{-4}$), hinders an accurate determination of the strain in those cases where the maximum strain component $\epsilon_{zz}(x,z)$ is below 1×10^{-4} . Better sensitivity could be achieved with better energy resolution by employing other existing monochromator schemes. Additional strain components could be obtained from the analysis of other crystallographic directions. An unprecedented lateral resolution of about 100 nm may be seen in Fig. 4b, where the ~ 10 experimental points associated with the narrow SiO₂ stripes show systematic variations of the strain between adjacent points, that is, lateral positions displaced by 110 nm at the sample surface. As we register all diffraction angles simultaneously, the overall measuring time for a complete rocking scan is only about 20 min. The method we describe here could be used on semiconductor devices in operating condition: it could also be applied to any crystalline sample, so could find applications in a variety of fundamental and industrially relevant problems. □

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Air entrapment in coatings by way of a tip-streaming meniscus

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Entrapment of small air bubbles is a problem for continuous liquid-film coatings processes. The coating of any surface requires that the surrounding air in contact with it be displaced by an advancing liquid interface. Studies of dynamic wetting suggest that if the interface motion is too rapid, the air is not completely removed and it becomes entrained in the coating material¹. This process, which can lead to undesirable flaws in the form of bubbles, blemishes or voids, limits the speed at which the substrate can be moved in the production of uniform precision coatings. However, the entrapment process is not understood in detail. Here we report an experimental investigation of air entrapment in high-speed coating operations. Tip streaming—a phenomenon well known in emulsification technology², involving the ejection of a fine filament from the cusped interface between two immiscible fluids—is shown to be the precursor of air entrainment. We demonstrate that tip-streaming air filaments emanating from the contact zone of a dynamic liquid interface give rise to minute ($\sim 10\ \mu\text{m}$) bubbles.

Considerable research has been carried out on air entrainment (see ref. 3 for a review). When the coating speed is less than a critical value for a particular material, the contact line is generally normal to the motion of the substrate. As the speed exceeds the critical value the contact line starts to become serrated. A qualitative explanation for this behaviour in the application of coatings for photographic film has been reported⁴. Other works^{5,6} have subsequently reported similar observations at supercritical coating speeds and all have noted that bubble generation originates at the leading-edge vertices of the serrations, that is, those projecting into the liquid.

A recent study of free-surface cusps⁷ noted that powder sprinkled on the liquid interface was swept into the interior of the fluid when the motion speed was sufficiently large. However, there was no evidence of air entrapment, so that the phenomenon was left

unexplained. That work was suggested by one⁸ that reported studies of two-dimensional cusps in a roller apparatus reminiscent of that originally used by Taylor⁹. An analogy between this type of flow and the motion of a dynamic contact line, however, is merely qualitative, because a cusp represents a singularity in the free-surface curvature and only occurs in the absence of surface tension⁸. In contrast, imposition of the no-slip boundary condition in the immediate vicinity of a dynamic three-phase contact line leads to a contradiction¹⁰ and it appears that a cusp cannot be formed without introducing slip at the boundary. Thus, although the contact-line interface appears to be cusp-like (see Fig. 1), analysis shows this not to be the case. Further analytical work on interfacial cusps has been reported recently^{11,12}.

Several works on nonlinear extensional flows, beginning with ref. 9, have reported that when a sheared drop becomes pointed, small bubbles can be ejected from the cusp. Tip streaming, however, only occurs when the viscosity ratio M of the two fluids involved is less than $O(0.1)$, see ref. 2. Here, $M = \mu_2/\mu_1$ and the subscripts 1 and 2 refer to the continuous phase and the dispersed phase, respectively. We note that this criterion is satisfied by air in combination with almost all liquids. Tip streaming is also discussed elsewhere^{13–16}. Other observations¹⁷ suggest that surfactants play some role in tip-streaming events, but it is unclear whether they are essential for the phenomenon to occur. Recently, analytical studies (Siegal, M, personal communication, and ref. 18) have examined the role of variable surface tension on unsteady cusp formation.

The basic experimental apparatus consists of a length of fibre that is pulled from a supply reel onto a take-up reel, passing through a two-dimensional, liquid-filled test cell in the process. Appropriately arranged pulleys position the fibre so that it is moving vertically downward as it enters the cell. Alignment of the fibre through the cell is achieved by micrometer adjustment on the pulleys above and below it. A stepper motor, controlled by a micro-step indexer, provides the drive to the take-up reel which moves the fibre at speeds of $0.01\text{--}3.5\ \text{m s}^{-1}$.

Two-dimensional test cells are constructed from $10 \times 10\text{-cm}^2$

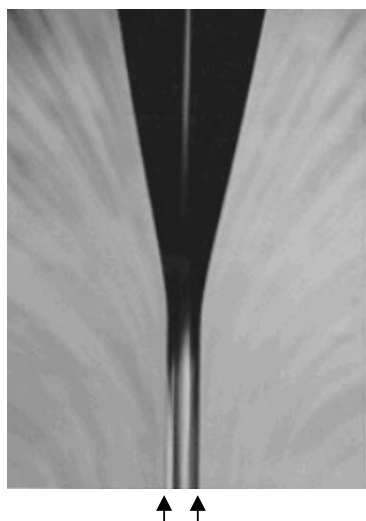


Figure 1 The air-liquid interface near the contact line on a $240\text{-}\mu\text{m}$ fibre. The fibre is moving vertically downward at $0.48\ \text{m s}^{-1}$; $Ca = 8.4$, $Re_d = 0.06$. The arrows indicate the edges of the fibre.

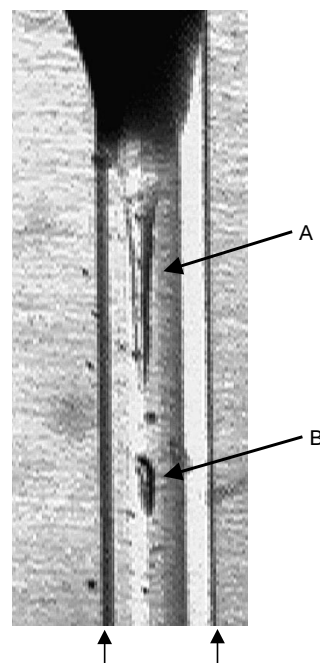


Figure 2 Close-up of the contact region. An air filament (A) extending from a plunging meniscus, and a separated bubble (B) are shown. The draw speed is $0.14\ \text{m s}^{-1}$; $Ca = 2.4$, $Re_d = 0.02$. The bubble is approximately $140 \times 40\ \mu\text{m}^2$. This high-speed image was recorded at framing rate of $500\ \text{s}^{-1}$ and the vertical field of view is about $1.3\ \text{mm}$. Arrows at the bottom of the image indicate the edges of the fibre.

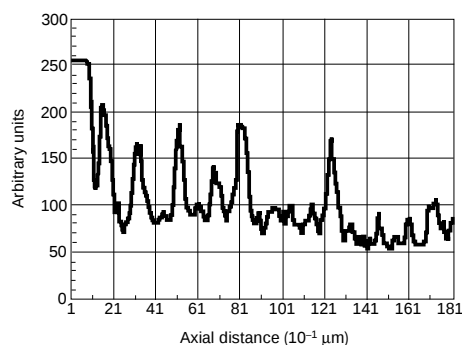


Figure 3 Representative bubble distribution from a tip-streaming meniscus. The mean diameter is $\sim 53 \mu\text{m}$ and the draw speed = 0.24 m s^{-1} ; $Ca = 4.2$, $Re_d = 0.03$.

panels of display glass separated by 2- or 4-mm thick Perspex spacers. With these configurations the sidewall influence on the observed motion was found to be negligible. The top of the cell, where the fibre enters the liquid, is open to the atmosphere and at the bottom the fibre leaves through a perforation in a silicone dam, which prevents excessive leakage. Observations of the air–liquid meniscus shape at the contact region with the fibre are made with a Nikon stereo microscope mounted horizontally on an adjustable traversing system. Typically, the field of view with this arrangement is $2 \times 2 \text{ mm}^2$ at a magnification of $\times 50$. A digital CCD (charge-coupled device) camera is used to obtain video recordings of the meniscus dynamics as a function of draw speed.

In all of the experiments we used fibre that is nominally $240 \mu\text{m}$ in diameter and has a urethane-acrylate exterior. The surface structure, viewed at $\times 1,000$ with a scanning electron microscope, appears to be smooth; any roughness is on a sub-micrometre scale. Before each test, we measured the fibre diameter with an optical caliper (Imagen HR 1024) coupled to a Nikon microscope. Fresh glycerine was used in all of the tests and the effect of water absorption has been neglected. The viscosity of the glycerine was recorded as $1,100 \text{ cP}$ at ambient temperature with a Brookfield DV-11 viscometer.

Bubble generation was first detected in the test cells at a draw speed of approximately 0.12 m s^{-1} . This speed corresponds to a critical capillary number $Ca_c (= \mu U / \gamma) \cong 2.1$. Here, μ is the viscosity, U is the draw speed and γ is the surface tension. The equivalent Reynolds number $Re (= \rho U d / \mu)$, where ρ is the density, based on the fibre diameter d , is $Re_d = 0.033$. This result corresponds to one particular density ratio, and variations of the liquid-phase properties are expected to modify the value of Ca_c . It has been suggested for a roll-coating system⁵ that an inertial group given by $\gamma(\rho/\mu^4 g)^{1/3}$, where g is the gravitational constant, is the other controlling parameter. Our result is in reasonably good agreement with that correlation.

Air bubbles as small as $6 \mu\text{m}$ have been detected in video recordings after the draw speed exceeds the critical value. At that point the meniscus is dragged progressively further into the liquid and fine filaments begin to appear at the unsteady contact line. Figure 1 shows the meniscus in close proximity to the fibre at a supercritical speed. The cusp-like distortion of the free surface under the shearing action of the fibre can be clearly detected. A tip-streaming filament that exists along the left side of the fibre near the apex of the meniscus is harder to discern. Streaks in the background of the picture indicate the trajectories of minute bubbles.

An enlargement of the dynamic contact region showing the interface under conditions close to critical is displayed in Fig. 2. In the image a distorted bubble which has just separated from the cusp is readily apparent. From our observations the bubbles do not

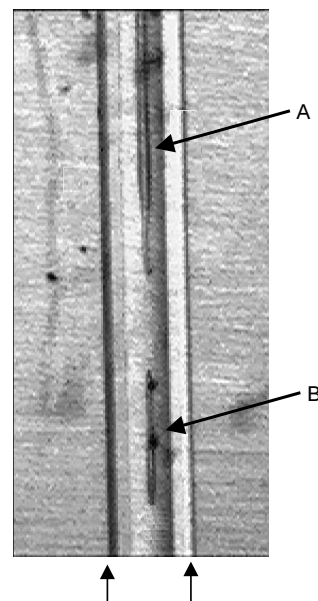


Figure 4 A tip-streaming filament (A) with an elongated bubble (B) on a fibre moving at 0.3 m s^{-1} ; $Ca = 5.0$, $Re_d = 0.04$. The framing rate is 500 s^{-1} . The dark spots are bubbles that are not in the focal plane. Arrows at the bottom of the image indicate the edges of the fibre.

seem to adhere to the fibre surface for very long; they migrate into the region about one fibre diameter from it under the action of vorticity. There is also some evidence to suggest that the extended meniscus itself is not always in contact with the fibre.

A line profile intensity trace taken through a series of bubbles issuing from a tip-streaming meniscus at $Ca = 4.2$ is shown in Fig. 3. The presence of six bubbles is easy to identify on this trace. Measurements of the bubble diameter on the photograph from which the trace was taken suggest a mean value of about $53 \mu\text{m}$, with a separation wavelength of approximately $150 \mu\text{m}$. The range of bubble diameters created at a particular value of Ca is difficult to assess because of the agglomeration that occurs during recirculation in the test cell. However, it does appear that close to Ca_c the bubble diameters are greater than those observed at larger capillary numbers.

Figure 4 displays the termination of a tip-streaming filament, again with a separated elongated bubble. This image, taken at a draw speed of 0.29 m s^{-1} ($Ca = 5.0$), illustrates how much the air filament can be extended by the shear flow. Here the filament length is at least eighteen times its width; an edge-enhancing software measurement suggests that the width is about $33 \mu\text{m}$.

These experiments demonstrate for the first time, to our knowledge, that coating flows at supercritical speeds give rise to tip-streaming air filaments. At the onset of air entrainment the critical capillary number is found to be approximately 2.1 for the glycerine–air case reported here. Tip streaming originates at the apex of the contact region interface and air bubbles are dispersed into the bulk of the liquid phase. Aspect ratios greater than twenty have been recorded for the air filaments observed in these experiments and their magnitude, although unsteady, appears to be to be speed dependent. A connection between air entrainment in coating operations and tip-streaming events has not, to our knowledge, been made previously. □

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Water exchange between the subglacial Lake Vostok and the overlying ice sheet

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It has now been known for several years that a 200-km-long lake, called Lake Vostok, lies beneath the ice sheet on which sits Vostok Station in Antarctica^{1–5}. The conditions at the base of the ice sheet above this subglacial lake can provide information about the environment within the lake, including the likelihood that it supports life². Here we present an analysis of the ice-sheet structure from airborne 60-MHz radar studies, which indicates that distinct zones of basal ice loss and accretion occur at the ice–water interface. Subglacial melting and net ice loss occur in the north of the lake and across its 200-km-long western margin, whereas about 150 m of ice is gained by subglacial freezing in the south. This indicates that significant quantities of water are exchanged between the base of the ice sheet and the lake waters, which will enrich the lake with gas hydrates, cause sediment deposition and encourage circulation of the lake water.

Three 60-MHz radar lines are aligned parallel to the general direction of ice flow, as indicated by interferometric-SAR (InSAR)

data (Fig. 1). These transects run across the northwestern margin of the lake (Fig. 2a), the southern central region of the lake (Fig. 2b), and from the southwestern margin of the lake to Vostok Station (Fig. 2c). InSAR data show the ice-velocity field above the lake, and along each of the transects, in metres per year (Fig. 1b). Several isochronous internal radar layers, extracted from the raw 60-MHz radar data, were traced across each transect. The vertical distance between the highest and lowest traceable internal layers, and that between the lowest layer and the ice-sheet base, were both then measured along these transects. The resulting data (Fig. 3) clearly indicate marked, spatially-coherent deviations in the dip of the basal radar layers away from parallelism with the ice-sheet base. Such deviations could be caused by a number of processes. For example, basal topography is commonly translated into the overlying ice column. However, such topography is negligible over Lake Vostok. The ice base in contact with the lake surface is characterized by a small (0.002) but steady gradient from the north of the lake where the ice is ~4,200 m in thickness, to the south where the ice is ~3,800 m thick. There is very little variation in ice thickness across the lake from west to east. This situation is therefore analogous to an ice shelf, where the absence of basal shear stress prevents deformation of internal layers. For the same reason, the influence of compressive flow, which would force internal layers apart, and extending flow, which would bring them closer together, is considered unlikely except near the lake margins, where an abrupt transition in basal drag is expected. Independent evidence for a relatively uniform stress field across the lake is provided by the InSAR data (Fig. 1b), which indicate a smooth divergence of ice, similar to that in floating ice shelves. Dipping internal layers could, however, still reflect oblique topographic influences inherited from grounded sections of the ice sheet located up-flow of the transect under consideration. In this case, the observed dip will vary with the sine of the angle between the transect and the ice flow direction. Inherited dip will therefore be zero where transects are aligned parallel to ice flow. Since no transect in the present study is aligned exactly parallel to the ice flow (Fig. 1), this argument cannot be used to constrain inherited layering precisely. However, the influence may be evaluated where two transects cross the same ice-flow path at different angles. Here, a similar pattern of inherited internal layering will be present in both transects, and the amplitude of the pattern will vary with the sine of the angle that each transect makes with the ice flow direction. Finally, basal ice melting and accretion as the ice sheet passes over the lake surface will cause flow-parallel internal layers to dip downwards and upwards respectively. We believe that at least three approximately flow-parallel transects indicate that such processes operate on a significant scale above Lake Vostok.

In transect AB when ice is grounded (Fig. 2a, Fig. 3a), four traceable internal layers are observed to run parallel to the ice sheet base, and are separated from each other by a relatively constant thickness along the transect. However, as ice flows over the western margin of the lake, the distance between the first and fourth internal layers increases by 200 m over a horizontal distance of 8 km, while that between the layer and the ice base decreases by 300 m (Fig. 3b). The rate of change of basal ice thickness with distance along the transect is greatest (–140 m km^{–1}) at 1 km downstream of the ice grounding line, decreasing to only a few metres per kilometre at 8 km from the grounding line (Fig. 3c). Since high basal relief is absent over the lake area, we interpret this thinning in terms of ice loss by basal melting. Here, the melt rate may be calculated from the InSAR-derived ice-surface velocities, since two-dimensional numerical modelling of ice flow over the lake indicates negligible change in horizontal ice velocity down the vertical ice column⁶. This model also calculates a vertical ice-velocity profile consistent with the dipping of our internal layers (Fig. 2a), where the thickness between layers increases down the flowline. Concurrent with this englacial thickening, the model predicts ice loss between the lowest layer and the ice base only if sub-ice melting is accounted for. The

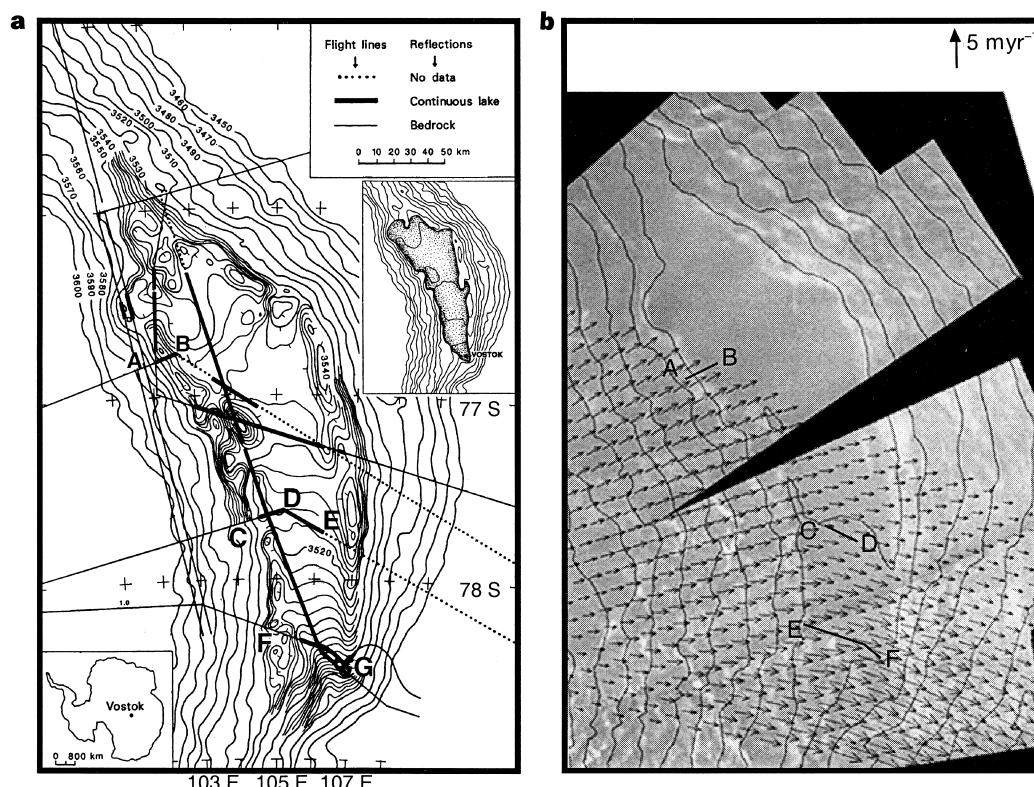


Figure 1 Surface elevation and velocity of the ice sheet above Lake Vostok, and positions of radar flightlines. **a**, ERS-1 satellite altimetry of the ice surface above Lake Vostok. The contour interval is 2 m over the lake, and is 10 m elsewhere. Tick marks on closed contour lines indicate downslope directions. The position of three 60-MHz airborne radar sounding transects (AB, CDE and FG) are illustrated. Also shown is the full series of radar flightlines around the lake, and the nature of the subglacial environment that these data record. The outline and location of Lake Vostok are provided in the insets². **b**, Ice motion derived from ascending and descending ERS tandem phase interferometry. The velocity vectors are

overlaid on an ERS mosaic of the ice-sheet surface. With the 70-day temporal baselines, the sensitivity of the interferometer to ice motion is better than 0.05 m yr⁻¹. Coherence in the target scattering over this long time interval is maintained because of the low accumulation rate at this elevation on East Antarctica. The data are referenced to a horizontally stationary point on the Ridge B ice divide. Previously, astronomical measurement of surface motion at Vostok in 1964 and 1972 gave 3.7 m yr⁻¹ at 142° true N. This velocity compares reasonably well with the interferometrically derived motion at Vostok Station of 4.2 m yr⁻¹ at 130° N.

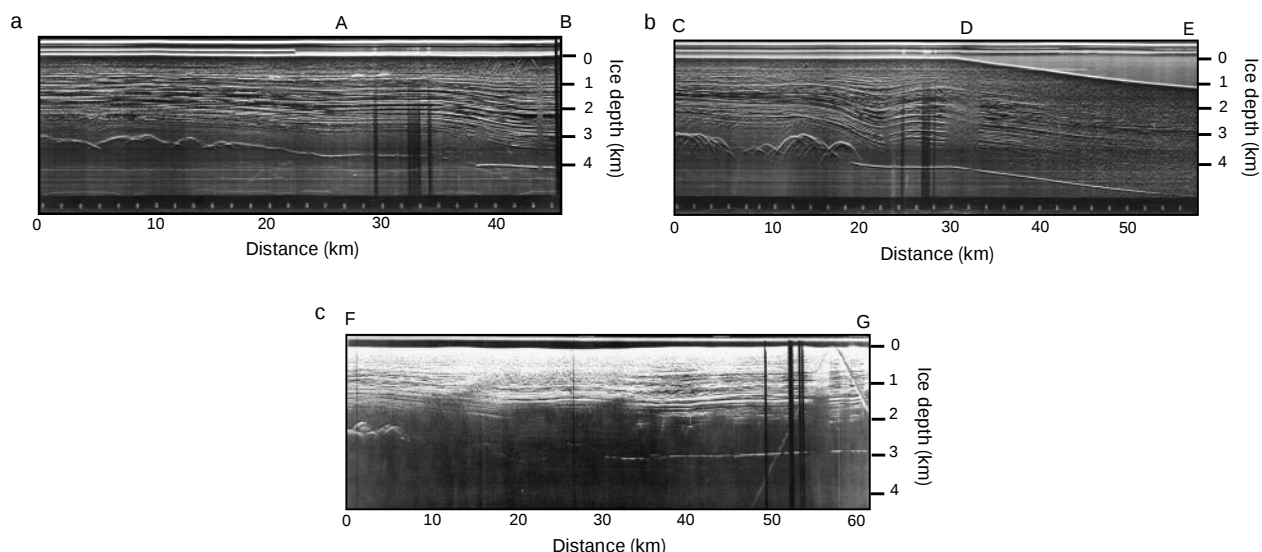


Figure 2 Airborne 60-MHz radar data used in the analysis of internal layering above Lake Vostok. **a**, Transect AB; **b**, transect CDE; **c**, transect FG (Fig. 1a). For AB and CDE, radar data 30 km upstream of the transects are shown to illustrate the glaciological setting of these transects. Internal ice-sheet structure is identified from internal layering. These internal layers are caused by ice-density changes to an ice depth no greater than 1 km; acidic layers of ice caused by aerosols from ancient volcanic emissions; and crystal orientation fabrics^{12,13}. Radar layers below 1 km of ice depth represent isochronous

surfaces and, near the ice-sheet base, are aligned along ice-particle flow paths⁶. We note that transect DE displays radar data recorded during the ascent of the aircraft. The radar reflections from the ice surface and ice base are maintained throughout this transect and demonstrate that variation in aircraft altitude does not adversely affect measurements of ice depth. In **c**, the location of Vostok Station is identified from the surface radar reflections off the station buildings at about 58 km along transect FG.

InSAR ice velocity is $\sim 2.7 \text{ m yr}^{-1}$ along transect AB, indicating that the melt rate reaches a maximum of $\sim 38 \text{ cm yr}^{-1}$ at 1 km downstream of the grounding line, and falls to $\sim 2 \text{ cm yr}^{-1}$ by 8 km from the grounding line.

In contrast to transect AB, net freezing of lake water to the ice base is indicated along the 22-km-long transect DE, in the central southern region of the lake. Internal layers are separated by a constant thickness from each other along this section (Fig. 3d and e). However, the thickness between layer 3 and the ice base increases by 250 m over 22 km, indicating a rate of ice gain of $\sim 10 \text{ m km}^{-1}$. The influence of inherited layering can be eliminated from this radar line, since it doglegs across the flow-line and patterns along transect CD differ markedly from those along transect DE (Fig. 2b). At an InSAR-derived ice-flow velocity of 2.2 m yr^{-1} , this gain corresponds with a mean freezing rate of 2.2 cm yr^{-1} . This evidence therefore indicates that basal ice is accreting over the central southern region of the subglacial lake.

Transect FG runs approximately parallel to ice flow from the southwestern margin of the lake to directly beneath Vostok Station (Fig. 1). As ice flows across the lake margin, the thickness between

the bottom layer and the ice base decreases by 150 m (Fig. 3g–i). This zone of net basal ice loss is coexistent with an increase in thickness between englacial layers of ice as in transect AB (Fig. 1a), and is interpreted as a region of basal melting. The regional InSAR ice velocity is 3.1 m yr^{-1} , indicating that the rate of subglacial melting is $\sim 6.5 \text{ cm yr}^{-1}$ along this 8-km-long section. In contrast, the ice thickness between the lowest layer and the ice base increases by 150 m over the next 8 km of the transect (Fig. 3h). The corresponding rate of basal ice accretion over this 8-km section, calculated with an InSAR velocity of 3.4 m yr^{-1} , is $\sim 6 \text{ cm yr}^{-1}$. Downstream from this freezing zone, the distance between the internal layers, and between the lowest layer and the ice base, both decrease. This pervasive thinning contrasts with that described above (which is largely restricted to the ice base), and occurs in an anomalous area of accelerating flow as the ice speeds up from 3.1 to 4.2 m yr^{-1} at Vostok Station (Fig. 1). This acceleration should theoretically yield 750 m of vertical thinning over the lake in transect FG. However, the observed amount of thinning is only 250 m. The extra 500 m of ice may be derived from surface and subglacial accumulation of ice. The rate of accumulation at Vostok Station is

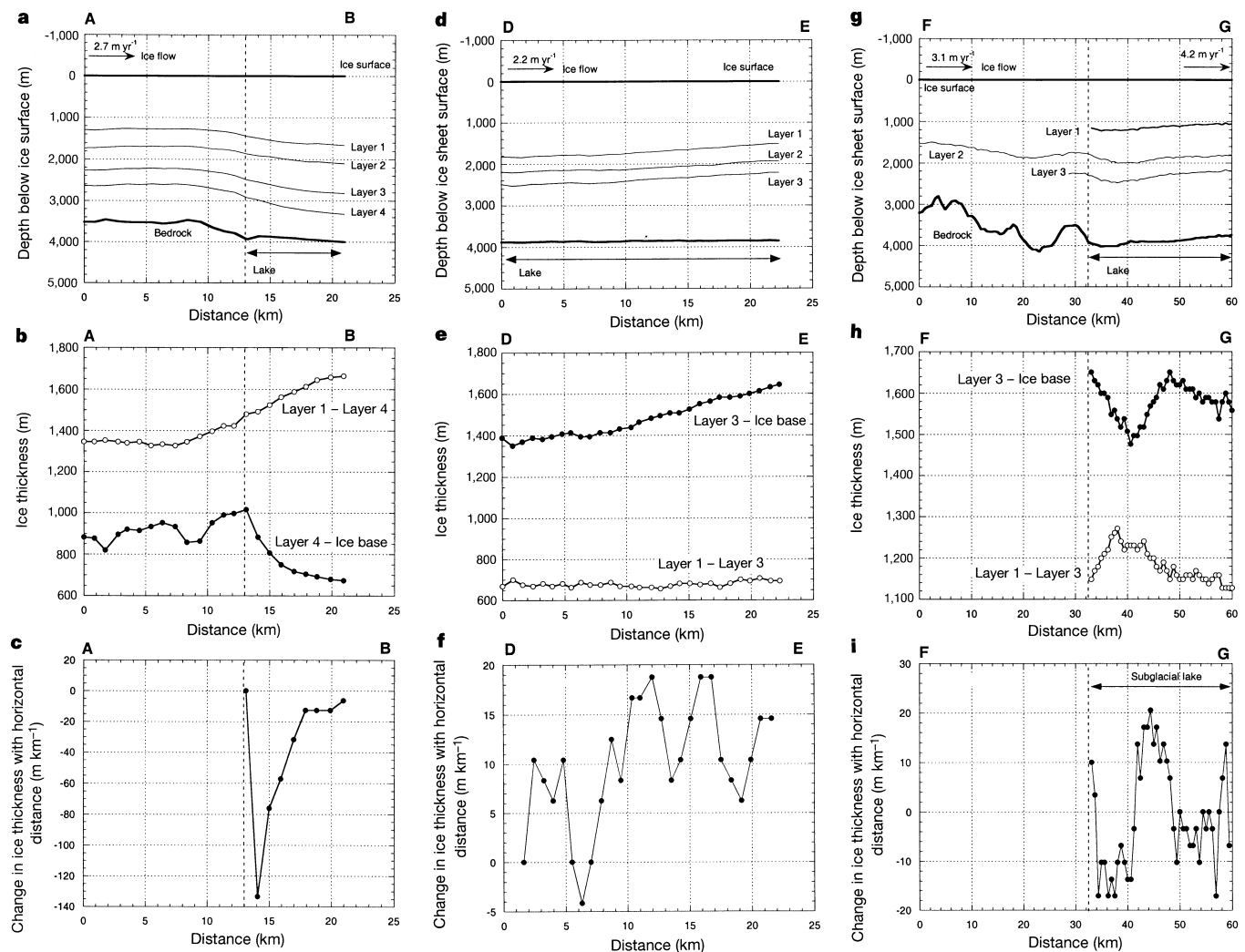


Figure 3 Analysis of internal layers extracted from airborne 60-MHz radar data from Lake Vostok. **a**, Processed radar data across transect AB. The depths below the ice surface of three internal layers and the ice-sheet base are shown. **b**, Ice thickness between internal layers 1 and 4 (open circles), and between layer 4 and the ice base (solid circles) across AB. **c**, Change in ice thickness between the fourth internal layer and the ice base with distance across AB. **d**, Processed radar data across transect DE. **e**, Ice thickness between layers 1 and 3 (open circles) and between the third internal layer and the ice base (solid

circles) across transect DE. **f**, Change in ice thickness between the third internal layer and the ice base with distance across transect DE. A three-point running average was used to smooth these data. **g**, Processed radar data across transect FG. **h**, Ice thickness between the first and third layers (open circles) and between the third internal layer and the ice base (solid circles) across transect FG. **i**, Change in ice thickness between the third internal layer and the ice base with distance across transect FG. A three-point running average was used to smooth these data.

~3 cm yr⁻¹, which over 5,000 years can supply less than half the required extra ice. This suggests that rates of subglacial freezing are higher than the rates of surface ice accumulation.

Our interpretation of flow-parallel radar data thus indicate net loss of basal ice along the western margin of Lake Vostok with melt rates ranging from >20 cm yr⁻¹ in the north to ~6 cm yr⁻¹ in the south. Further, in the southern central regions of the lake, net freezing of water causes build-up of accreted ice at a rate of between ~2 and ~6 cm yr⁻¹. In the two cases where our analysis shows basal freezing, the rate of ice freezing increases toward the south of the lake. Thus, the ice–water interface above Lake Vostok is characterized by melting along the western margin of the lake, with greatest melt rates in the north, and subglacial freezing in the centre of the lake, with greatest freezing rates around the south. Significantly, independent evidence for this interpretation is provided by the crystallographic and included gas properties of the Vostok ice core, which indicate that 200 m of ice has been accreted on to the base of the ice sheet from the lake⁷. Our under-prediction of the total accreted ice thickness at Vostok Station by 50 m may be due to processes other than subglacial freezing, detailed above, providing a context within which the accuracy of our melting and freezing rates should be viewed.

Basal melting and refreezing above Lake Vostok will encourage lake water circulation as has been observed beneath large ice shelves⁸. Further, there will be a net transfer of impurities from the influent basal ice to the lake water. In the East Antarctic Ice Sheet, these impurities include gas hydrates^{9,10}, dissolved ionic species¹¹ and, almost certainly, debris. Although these impurities would be transferred to Lake Vostok water upon melting, it is extremely unlikely that they would be transferred in the same concentrations back to the relatively pure refrozen ice above the lake. Our data therefore indicate a net transfer of these materials to Lake Vostok waters, with important implications not only for the sedimentology and chemical evolution of the lake, but also for the potential survival of living organisms within the lake. □

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Stable-isotope probing as a tool in microbial ecology

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Microorganisms are responsible for driving the biogeochemical cycling of elements on Earth. Despite their importance and vast diversity¹, the taxonomic identity of the microorganisms involved in any specific process has usually been confined to that small fraction of the microbiota that has been isolated and cultivated. The recent coupling of molecular biological methods with stable-isotope abundance in biomarkers has provided a cultivation-independent means of linking the identity of bacteria with their function in the environment^{2,3}. Here we show that ¹³C-DNA, produced during the growth of metabolically distinct microbial groups on a ¹³C-enriched carbon source, can be resolved from ¹²C-DNA by density-gradient centrifugation. DNA isolated from the target group of microorganisms can be characterized taxonomically and functionally by gene probing and sequence analysis. Application of this technique to investigate methanol-utilizing microorganisms in soil demonstrated the involvement of members of two phylogenetically distinct groups of eubacteria; the α-proteobacterial and *Acidobacterium* lineages. Stable-isotope probing thus offers a powerful new technique for identifying microorganisms that are actively involved in specific metabolic processes under conditions which approach those occurring *in situ*.

Methylotrophs are a specialized group of microorganisms that can utilize reduced carbon substrates with no carbon–carbon bonds as a sole source of carbon and energy⁴. Growth of the methylotrophic bacterium *Methylobacterium extorquens* on the carbon source ¹³CH₃OH (99% ¹³C) resulted in an increase in the buoyant density of DNA relative to that from *M. extorquens* grown on ¹²CH₃OH, which could be easily resolved by density-gradient centrifugation (Fig. 1a). A similar degree of separation between 'light' (¹²C) and 'heavy' (¹³C) DNA was achieved when a methanotroph, *Methylomicrobium album*, was grown on ¹²CH₄ or ¹³CH₄ (99% ¹³C), as would be expected for any pure culture using a uniformly ¹³C-labelled compound as a sole carbon source. When *M. extorquens* was grown on deuterium-labelled methanol (CD₃OD; 99.8% D), isotopically labelled and unlabelled DNA bands were also obtained, but their separation was ~50% of that achieved with ¹³CH₃OH as the substrate. The removal of two deuterium atoms during oxidation of CD₃OD to DCDO and the subsequent isotopic exchange during assimilation through the serine pathway⁵ can explain the observed reduction in buoyant density. Incorporation of a ¹⁵N-labelled substrate as a sole nitrogen source would also be expected to increase the DNA buoyant density, as has been shown for the semi-conservative replication of DNA⁶. This ability to isolate DNA from microorganisms that have assimilated a specific growth substrate prompted us to assess the potential of using stable-isotope-enriched substrates for the identification of metabolically active members within a complex natural environment.

Using traditional microbiological methods, the identification of physiologically distinct groups of microorganisms first requires their isolation into pure culture. Here we used a cultivation-independent method for linking function with taxonomic identity, which involves the application of stable isotopes to probe the DNA

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of metabolically active microorganisms in an environmental sample. A ^{13}C -labelled DNA fraction obtained from microorganisms utilizing $^{13}\text{CH}_3\text{OH}$ in an oak forest soil (Fig. 1b) was purified from unlabelled DNA (Fig. 1c). When DNA (^{12}C) from the corresponding soil exposed to $^{12}\text{CH}_3\text{OH}$ was used as a template for the polymerase chain reaction (PCR), a specific product was obtained with primer sets that amplify eubacterial, archaeal and eukaryal small-subunit ribosomal RNA genes. However, PCR amplification with ^{13}C -DNA from the soil exposed to $^{13}\text{CH}_3\text{OH}$ indicated that only eubacteria were involved in methanol assimilation. Phylogenetic analysis of 16S ribosomal DNA sequences amplified from the ^{13}C -DNA (Fig. 2) showed that bacteria within two distinct lineages, the α -subclass of the Proteobacteria and the *Acidobacterium* division, had assimilated the CH_3OH . The α -proteobacterial clones UP1, UP2 and UP3 were closely related to the 16S rRNA genes of *Beijerinckia indica*, *Rhodopseudomonas acidophila* and eubacterium K (ref. 7). The remaining three clones, UA1, UA2 and UA3, were grouped with 16S rRNA gene sequences from phylogenetic group I of the *Acidobacterium* division⁸. Although *Acidobacterium capsulatum* is one of only three species within this division that has been cultivated and described to date, 16S rDNA sequence studies indicate that a diverse range of phylogenetically related bacteria exist in many environments¹. It has been suggested that this abundance of *Acidobacterium*-related 16S rDNA sequences indicates a potential for the division to be physiologically versatile⁸ and to have a significant role in biogeochemistry¹. We provide direct evidence that certain members of the *Acidobacterium* division can indeed perform the same function (methanol assimilation) as bacteria from several other divisions.

Methanol can be metabolized and assimilated by a wide range of microorganisms, including Gram-negative and Gram-positive bacteria, and several yeasts⁹. It was unexpected, therefore, that the methylotrophs that we detected were confined to only two phylogenetic groups and were most closely related to genera that are rarely associated with methanol utilization. It is possible that the relatively high methanol concentration (0.5% v/v), coupled with the acidity of the forest soil (pH 3.5), provided conditions more suitable for the growth of unusual methylotrophs. Indeed, the bacteria in culture that are phylogenetically most closely related to the clone sequences grow at acidic pH (refs 10, 11). Bacteria with 16S rRNA sequences similar to those of both *Beijerinckia* and *Acidobacterium* have also been shown to be active in an acidic soil (pH 4–5)¹². Although the 16S rDNA clone library was dominated by α -proteobacterial-related sequences, biases inherent in PCR mean that we cannot estimate the relative abundance of the bacteria represented by these sequences. It is equally important to stress that the absence

of sequences from more typical methylotrophs does not preclude the involvement of these microorganisms in methanol metabolism in this forest soil.

Genes that encode key enzymes in metabolic pathways ('functional genes') have been used to identify microbial groups with a known metabolic function. Using this approach, new functional gene sequences detected in environmental samples have been tentatively linked with the identity of the microorganisms containing these sequences^{13,14}. The enrichment of a limited number of genomes in the ^{13}C -DNA fraction provided an opportunity to link directly functional gene sequences with a newly identified group of methylotrophs. We used PCR primers that amplify part of *mxoF*, the gene that codes for the α -subunit of the methanol dehydrogenase found in all Gram-negative methylotrophic bacteria⁴. Three new, but related, *mxoF* sequences (M13.1–M13.3) were amplified from the ^{13}C -DNA (Fig. 3). As the phylogenetic relationships between *mxoF* sequences of known strains recover clusters corresponding to the α -, β - and γ -subclasses of the Proteobacteria¹⁴, it is highly probable that the new *mxoF* sequences were from one or more of the microorganisms represented by UP1–UP3. Furthermore, this parallel analysis of *mxoF* gene sequences supported the 16S rDNA analysis that indicated that the active Gram-negative methylotrophs in the forest soil were restricted to a new group in the α -subclass of the Proteobacteria.

In recognition of the need for a cultivation-independent means of linking the functional activities of microorganisms with their taxonomic identity, several innovative methods have been developed^{2,15,16}. Two approaches with broad applicability include the combination of fluorescent *in situ* hybridization (FISH) with microautoradiography^{17,18} and the coupling of radio- or stable-isotope analyses with detection of microbial phospholipid fatty acids (PLFAs)^{3,19}. The stable-isotope probing (SIP) method that we used is analogous to these methods in that it relies on the incorporation of an isotope with a low natural abundance into components of the biomass (in this case the DNA) of a microorganism utilizing an isotopically enriched substrate. However, the ability to isolate an entire copy of the genome of microorganisms involved in the metabolism of a substrate makes SIP unique and provides advantages over other methods when attempting to link a metabolic activity with taxonomic identity. Of most value is the confidence with which a DNA sequence can be assigned to a taxonomic clade. This feature is particularly important when several uncultivated microorganisms are involved in a process, as we detected for methanol utilization. In such cases, PLFA analyses may result in a composite profile that is unlike that of any single organism. Similarly, probes used for FISH only target small regions of the rRNA, which may restrict their specificity¹⁸ and make

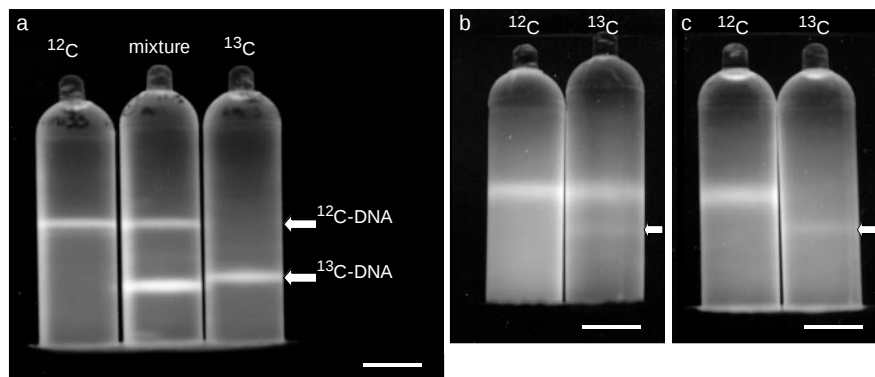


Figure 1 Equilibrium centrifugation of isotopically labelled DNA in CsCl/ethidium bromide density gradients. **a**, Pure fractions and a mixture of the DNA extracted from a *M. extorquens* AM155 culture utilizing either ^{12}C - or ^{13}C -methanol as the sole carbon source.

b, DNA extraction from soil that had utilized ^{12}C - or ^{13}C -methanol, showing the position of the ^{13}C -DNA fraction (arrow) after the primary and secondary, **c**, purification ultracentrifugation steps. Bar, 1 cm.

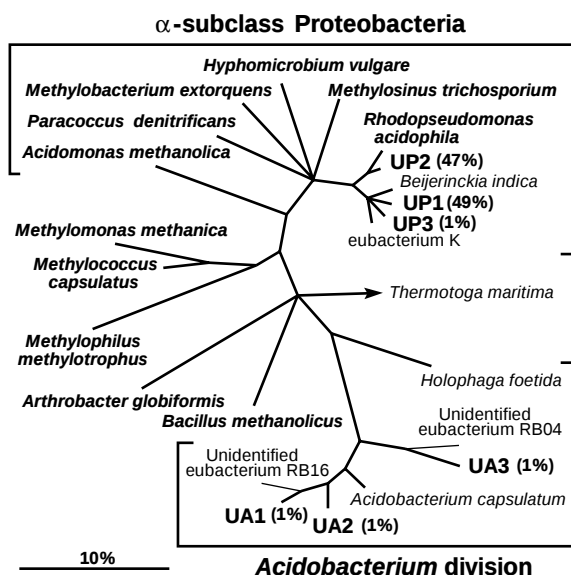


Figure 2 Phylogenetic analysis of bacterial 16S rRNA gene sequences amplified from ^{13}C -DNA. Bold text indicates genera known to use methanol. Analysis was based on 1,204 nucleotide positions. Percentage of the library represented by each clone type is in brackets. *Thermotoga maritima* was used as an outgroup. Sequences with less than 1,400 bases (thin lines) were added to the consensus tree with a maximum-parsimony method. Scale bar, 10 inferred substitutions per 100 nucleotide positions.

taxonomic identification difficult. Another valuable feature of SIP is that it provides access to the genes involved in a function. This will allow the application of molecular biological techniques for identification of potentially new pathways, such as that for methanol assimilation in the *Acidobacterium*-like organisms.

The SIP approach has potential limitations, especially in relation to sensitivity, which may render the technique unsuitable in certain situations. Perhaps the single most important factor that might affect the identification of the target microorganisms is the dilution of a labelled substrate before its assimilation and incorporation. In the case of carbon, simultaneous growth on an unlabelled (^{12}C) substrate will dilute the proportion of ^{13}C that is incorporated into DNA. This will reduce the proportion of DNA that becomes isotopically labelled, making it less easy to target those microorganisms that are primary users of a substrate. Unlabelled carbon sources may include another organic compound or the same substrate produced by internal processes in the environment being investigated²⁰. A second important factor is the fate of the isotopically labelled atom of a ^{13}C -labelled substrate. Microbial metabolism of a ^{13}C -labelled substrate may also lead to the incorporation of ^{13}C into products and intermediates of metabolism, which might be assimilated by other, non-primary, organisms. Production of the corresponding ^{12}C -labelled compounds and trophic interactions would, however, dilute these modified substrates in a complex environment such as soil. DNA bands in the soil-labelling experiment were separated by 65% as compared with pure cultures, which could be attributed to isotopic dilution. With further development of SIP, we estimate that as little as 20% incorporation of a ^{13}C -labelled substrate into DNA will be sufficient to resolve ^{13}C -DNA from ^{12}C -DNA and maintain, with confidence, a link between identity and function.

We believe that SIP has the potential for wide application in microbial ecology. It offers a cultivation-independent means of investigating the effect of changes in environmental conditions, including temperature, water potential, pH and substrate concentrations, on the taxonomic identity of functionally active groups of

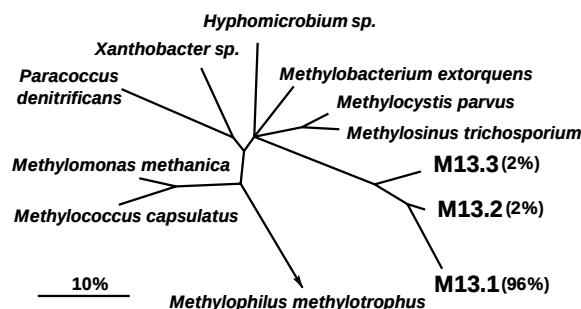


Figure 3 Phylogenetic analysis of deduced amino-acid sequences corresponding to the *mxoF* genes amplified from ^{13}C -DNA. Analysis was based on 171 amino-acid positions. Percentage of the library represented by each clone type is in brackets. The *MxaF* sequence of *M. methylotrophus* was used as an outgroup. Scale bar, 10 inferred substitutions per 100 amino-acid positions.

microorganisms. This ability to link function with taxonomic identity may also assist in the formulation of isolation strategies for microorganisms that are actively involved in ecologically important processes. With an ever-increasing range of ^{13}C -enriched substrates becoming available, and the potential of using ^{15}N - and ^2H -enriched compounds, it will be possible to use SIP to identify many microorganisms involved in the cycling of carbon and nitrogen in the environment. □

Methods

DNA labelling, extraction and fractionation

Methylobacterium extorquens AM155 and *M. album* BG8 were grown at 30°C in ANMS medium²¹ supplemented with CH_3OH (0.5% v/v) or CH_4 (10% v/v), respectively. DNA was extracted from 10 ml of a stationary phase culture²² and purified by ultracentrifugation in a small-scale (13×51 mm tubes; Beckman) CsCl/ethidium bromide density gradient²³. An oak (*Quercus petraea*) forest soil (0–5 cm depth) collected from the Gisburn Forest Experiment²⁴ was exposed to methanol in a microcosm experiment. The soil was sieved and air-dried to ~35% water holding capacity (w.h.c.). Two separate 10-g samples were transferred into 126-ml crimp-top serum vials and sealed with grey rubber stoppers. Drop-wise addition of 0.5 ml of a ^{13}C - or ^{12}C -methanol solution (10% v/v in water) increased the final soil moisture to 40%–50% w.h.c. Soils were incubated at 25°C and the headspace gas samples were monitored by gas chromatography-flame ionization detection every 2–5 days. Twice during each incubation period, after a decrease of 25–40% of the initial methanol concentration, the vials were opened, flushed with 250 ml of air to ensure that the microcosms remained fully aerobic and resealed. When the methanol had been entirely consumed ($^{13}\text{CH}_3\text{OH}$, 18 days; $^{12}\text{CH}_3\text{OH}$, 24 days) the vials were opened, flushed with a gentle stream of air to reduce the soil moisture content to ~35% w.h.c. and resealed. A second drop-wise addition of the respective methanol solution to each microcosm re-established the methanol concentration at 0.5% (v/v) and the soil moisture at 40–50% w.h.c. After 44-days incubation, when 2.4 mmol of methanol had been consumed, a bead-beating method²⁵ was used to extract DNA from 1 g or 0.3 g of soil exposed to ^{13}C - or ^{12}C -methanol, respectively. CsCl was added to the DNA eluted from the glassmilk at a final concentration of 1 g ml^{-1} . After equilibrium centrifugation in small-scale CsCl/ethidium bromide gradients, DNA fractions were collected with a syringe and needle. In the case of the ^{13}C -methanol exposed soil, care was taken to avoid the ^{12}C -DNA band during collection of the ^{13}C -DNA fraction. A second ultracentrifugation step was carried out to increase the isotopic purity of the ^{13}C -DNA fraction. Collection of the ^{13}C -DNA fraction from the purification gradient removed any trace of contaminating ^{12}C -DNA withdrawn during the primary fraction collection. DNA fractions were treated with butanol to extract ethidium bromide, dialysed and resuspended in TE buffer.

DNA sequencing and analysis

The ^{12}C - and ^{13}C -DNA fractions collected from ^{12}C - and ^{13}C -methanol exposed soils, respectively, were used as templates for PCR. Primers specific for bacterial, archaeal and eukaryal small-subunit rRNA genes²⁶ (~1,500 base-pair product) and *mxoF*¹⁴ (~550 base-pair product) were used as described. Amplification products were cloned with the TOPO TA cloning kit (Invitrogen) and libraries of 100 clones (eubacterial 16S rDNA) or 50 clones (*mxoF*) were constructed. Plasmid inserts were screened by digestion with restriction endonucleases: *EcoRI*/*RsaI* (16S rDNA) or *EcoRI*/*HincII* (*mxoF*). At least 10% of clones within each restriction pattern were partially sequenced (>400 bases) to

verify that the profile represented a single clone type. Complete sequences were obtained for each unique restriction pattern. Before translation to the deduced amino-acid sequence, cloned *mxhA* sequences were screened for chimerae by visual comparison with the *mxhA* sequences of extant strains. Sequence alignment and phylogenetic analyses were done with the ARB programme (<http://www.mikro.biologie.tu-muenchen.de>), omitting regions of sequence ambiguity and incomplete sequence from the analyses. Results were depicted as a consensus tree, combining the results of evolutionary distance (Jukes and Cantor model for DNA; Dayhoff PAM model for amino acids), maximum-parsimony and maximum-likelihood analyses of the datasets. Multifurcations indicate points where the branching order was not supported by all three methods.

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Correspondence and requests for materials should be addressed to J.C.M. (e-mail: cm@dna.bio.warwick.ac.uk). The clone nucleotide sequences (clones UP1–UP3, UA1–UA3 and M13.1–M13.3) have been deposited in GenBank under accession numbers AF200694–AF200702.

Ancestral chloroplast genome in *Mesostigma viride* reveals an early branch of green plant evolution

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Sequence comparisons suggest that all living green plants belong to one of two major phyla^{1–3}: Streptophyta⁴ (land plants and their closest green algal relatives, the charophytes); and Chlorophyta⁵ (the rest of green algae). Because no green algae are known that pre-date the Streptophyta/Chlorophyta split, and also because the earliest diverging green algae show considerable morphological variation, the nature of the unicellular flagellate ancestor of the two green plant phyla is unknown^{1,6,7}. Here we report that the flagellate *Mesostigma viride* belongs to the earliest diverging green plant lineage discovered to date. We have sequenced the entire chloroplast DNA (118,360 base pairs) of this green alga and have conducted phylogenetic analyses of sequences derived from this genome. *Mesostigma* represents a lineage that emerged before the divergence of the Streptophyta and Chlorophyta, a position that is supported by several features of its chloroplast DNA. The structure and gene organization of this genome indicate that chloroplast DNA architecture has been extremely well conserved in the line leading to land plants.

Mesostigma is a scaly, green biflagellate that belongs to the Prasinophyceae⁸, a morphologically heterogeneous class that includes descendants of the earliest diverging green algae^{2,7–9}. No unique character unites the Prasinophyceae to the exclusion of other green algal classes. In phylogenetic trees inferred from nuclear small subunit (SSU) ribosomal DNA^{1–3,7,9} and actin-coding sequences¹⁰, all prasinophytes examined so far, except *Mesostigma*, form independent lineages at the base of Chlorophyta. *Mesostigma* represents the earliest divergence within Streptophyta: in SSU rRNA trees¹, this position is supported by low bootstrap values (52%), whereas in actin-gene trees¹⁰, it is more strongly supported (>81%). A specific affinity between *Mesostigma* and charophytes was previously predicted on the basis of the identical orientation of multilayered structures relative to flagellar roots^{1,7,11}.

The 135 genes in *Mesostigma* chloroplast DNA (cpDNA) (Fig. 1) represent the largest gene repertoire ever reported among green algal and land plant cpDNAs. We analysed the concatenated protein sequences (10,629 amino-acid positions) from 53 genes that are common to the cpDNAs of *Mesostigma*, three land plants (*Marchantia polymorpha*, *Pinus thunbergii* and *Nicotiana tabacum*) and three chlorophyte green algae (*Nephroselmis olivacea*, *Chlorella vulgaris* and *Pedinomonas minor*). The homologous proteins from the glaucocystophyte *Cyanophora paradoxa*, the red alga *Porphyra purpurea* and the cyanobacterium *Synechocystis* sp. PCC6803 were used to root the green algal phylogeny in these analyses. It is generally accepted that all chloroplasts were derived from a single primary endosymbiotic event involving the capture of a cyanobacterium¹². The chloroplasts of glaucocystophytes, red algae and green algae are thought to be direct products of this primary endosymbiotic event¹². Phylogenetic evidence indicates that glaucocystophyte chloroplasts evolved before those of red and green algae¹³.

Trees constructed with distance, maximum parsimony and maximum likelihood inference methods using *Cyanophora* proteins as an outgroup were found to be congruent in showing a strongly supported topology, 'T1' (Fig. 2a), in which *Mesostigma* emerges before the divergence of Streptophyta and Chlorophyta. Only T1

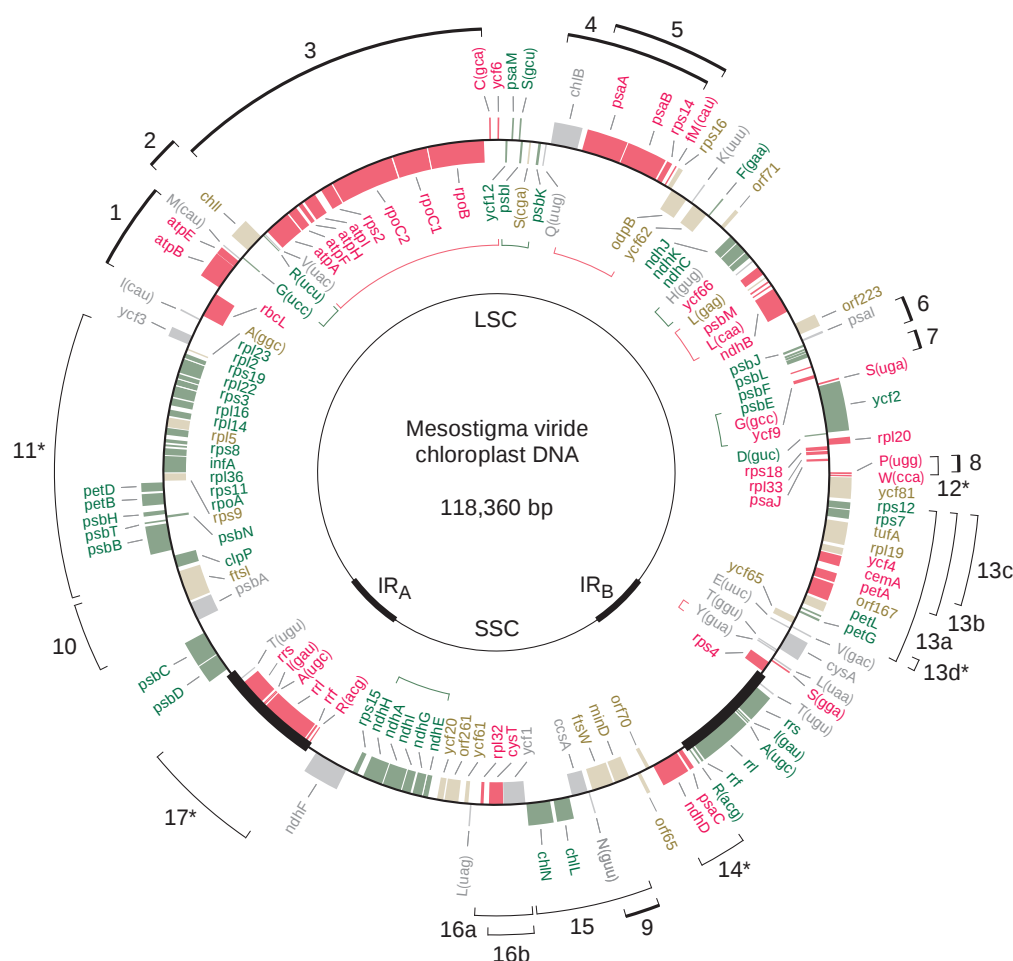


Figure 1 *Mesostigma* cpDNA. Inverted repeat sequences (IR_A, IR_B) separate small (SSC) and large (LSC) single-copy regions. Genes outside the map are transcribed clockwise. Genes absent from *Marchantia* cpDNA are represented in beige. Gene clusters shared with *Marchantia* are shown as series of green and red boxes; those corresponding to brackets inside the map are shared exclusively with land plants. Genes present in

Marchantia but located outside conserved clusters are shown in grey. Thick brackets indicate clusters that are shared with some non-green algae but are absent from *Synechocystis*. Thin brackets denote clusters that are shared specifically with both chlorophytes and streptophytes (asterisks) or only chlorophytes.

was detected in quartet-puzzling and distance-based analyses under the amino-acid substitution models of Jones *et al.*¹⁴ (JTT-F) and Dayhoff *et al.*¹⁵, with either a uniform or a gamma-distributed rate of substitution across sites. In maximum-parsimony analysis, bootstrap support for T1 was 97%, and two alternative topologies, 'T2' and 'T3' (Fig. 2b), differing with respect to the position of *Mesostigma*, were each recovered in 1.5% of the bootstrap samples. T1, T2, and T3 were also the only topologies recovered in maximum-likelihood analysis under the JTT-F model with a uniform rate of substitution; T1 was found in 98.9% of bootstrap samples, and both T2 and T3 were significantly worse than T1 (T2, $P < 0.05$; T3, $P < 0.01$) in the Kishino-Hasegawa test¹⁶. Removal of constant sites from the data set had negligible effect on bootstrap support for T1 in distance and maximum-likelihood analyses, and on the confidence limit of tree topologies under the Kishino-Hasegawa test (see Supplementary Information). Considering that most constant sites (5,585 out of 5,628) were estimated to be invariable using SPLITSTREE, these observations eliminate the possibility that misleading effects of invariable sites¹⁷ contributed to the recovery of T1. Even when the *Cyanophora* sequences were excluded from the data set, or when other outgroup sequences (*Porphyra* proteins, or a combination of *Cyanophora*, *Porphyra* and *Synechocystis* proteins) were used, trees compatible with T1 remained the best topologies (see Supplementary Information).

Systematic biases in amino-acid composition can give rise to incorrect relationships in reconstructed phylogenetic trees¹⁸. Chi-squared analysis of amino-acid composition at variable sites reveals that the 53 concatenated proteins of *Mesostigma* do not significantly differ ($P < 0.05$) from those of *Cyanophora*, *Marchantia*, *Pedinomonas* and *Pinus*. Our failure to detect a systematic bias in amino-acid composition thus suggests that the basal position of *Mesostigma* is the result of a genuine phylogenetic signal. In support of this conclusion, neighbour-joining analysis of LogDet distances calculated after removal of all constant sites retrieved the T1 topology in 93% of bootstrap samples, with T2 being the only alternative topology detected. LogDet distances allow the recovery of the correct tree when sequences differ markedly in amino-acid frequencies for cases when substitution processes are otherwise uniform across the underlying tree¹⁹.

We also carried out maximum-likelihood analyses of concatenated chloroplast SSU and large subunit (LSU) rDNA sequences (4,016 positions, of which 1,026 are variable) from the taxa shown in Fig. 2, using the HKY model of nucleotide substitution¹⁶ (for analyses with other models, see Supplementary Information). The reconstructed trees were found to be congruent with those inferred from chloroplast proteins in providing unequivocal support for T1. T1 and T3 accounted for 99.0% and 1.0% of bootstrap samples, respectively (T2 was not recovered), and T3 was significantly worse

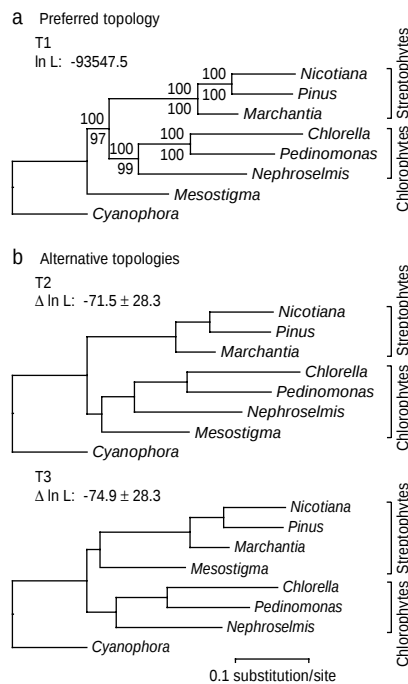


Figure 2 Phylogenetic position of *Mesostigma* as inferred from 53 chloroplast proteins. **a**, Preferred tree topology found in all analyses. The log likelihood (ln L) value was calculated using PROTML and the JTT-F model. The numbers above the nodes indicate the bootstrap percentages and reliability percentages obtained in distance and quartet-puzzling analyses, respectively. The numbers below the nodes indicate the bootstrap percentages in maximum-parsimony analysis. **b**, Alternative tree topologies detected in maximum-likelihood, maximum-parsimony and LogDet analyses. The Δ lnL value indicates the difference in log likelihood relative to T1 and the standard error of that difference.

than T1 ($P < 0.05$) in the Kishino-Hasegawa test. Removal of all constant sites from the data had little effect on support for T1.

In favouring the placement of *Mesostigma* within Streptophyta (a position corresponding to T3), actin-gene trees¹⁰ contrast with those that we inferred from chloroplast sequence data. We used the Kishino-Hasegawa test under the HKY model to assess the confidence limit of actin-tree topologies (see Supplementary Information) and found that the phylogenetic information in the actin data set (198 variable sites) is not sufficient to eliminate the hypothesis that *Mesostigma* branches at the base of Chlorophyta and Streptophyta (T1). The actin trees that support this hypothesis represented a meaningful proportion (12.7%) of bootstrap samples in maximum-likelihood analysis and were not significantly worse ($P \geq 0.30$) than those compatible with T3 (best maximum-likelihood trees). From this result, we conclude that the discrepancy between the chloroplast and the actin tree is only apparent.

The positioning of *Mesostigma* as the earliest divergence in the phylogeny of green plants is supported by our finding that this prasinophyte has retained more ancestral cpDNA features than previously examined green plants. The most relevant of these ancestral features are (1) a quadripartite structure in which common genes reside in corresponding genomic regions—a feature that is also shared with the cpDNAs of some non-green algae, most land plants and *Nephroselmis*²⁰; (2) the presence of five genes (*trnA*(ggc), *odpB*, *ycf20*, *ycf61* and *ycf65*) that were previously identified only in *Porphyra* cpDNA; (3) the presence of all genes that were lost specifically in Chlorophyta or Streptophyta²⁰, with the exception of one (*rpl21*) of the eight genes specifically missing from Chlorophyta and three (*rne*, *rnpB* and *rpl12*) of the sixteen genes

missing from Streptophyta; (4) the presence of nine gene clusters that are found in some non-green algal cpDNAs but not in the *Synechocystis* genome (Fig. 1)—only subsets of these clusters have been retained in other green plants (Table 1); and (5) the absence of introns—a feature that is also shared with the cpDNAs of *Nephroselmis*²⁰ and most non-green algae¹².

Many genes in *Mesostigma* cpDNA form clusters that are shared exclusively with chlorophytes and/or streptophytes (Table 1), thus strengthening the notion that *Mesostigma* represents the most basal green plant lineage. These clusters provide clues into how the green plant chloroplast genome diverged from the ancestral pattern of gene organization during the evolution of Chlorophyta and Streptophyta.

The gene organization of *Mesostigma* cpDNA is highly similar to that of land plant cpDNAs, with 81% of its genes being found in clusters that are shared with land plant cpDNAs (Fig. 1). This observation indicates that the chloroplast genome from the common ancestor of chlorophytes and streptophytes has been highly preserved in structure and gene order during the long evolutionary period (~800 Myr (ref. 6)) separating this ancestor from land plants. Such an exceptional conservation of gene order was unexpected, as *Nephroselmis* cpDNA, the green algal cpDNA previously known to display the most ancestral characters, shares a limited number of gene clusters with its land plant counterparts²⁰. Our results predict that cpDNA rearrangements occurred relatively infrequently in Streptophyta and that analysis of these events will be useful not only to study how the chloroplast genome evolved during the transition from the most ancestral green flagellate to land plants, but also to clarify phylogenetic relationships among streptophytes.

The morphological characteristics⁸ of *Mesostigma* support the view that the most ancestral green flagellate was a biflagellated and asymmetric cell that had an underlayer of square scales (a character unique to green algae), an eye spot and a cruciate flagellar root system with multilayered structures^{1,7}. The number of flagella is highly variable among prasinophytes and other green algae. Square-shaped scales are found in members of both Chlorophyta and Streptophyta; however, the presence of an eye spot has been noted

Table 1 Distribution of the *Mesostigma* chloroplast gene clusters that are shown in Figure 1

Cluster	Non-green algae				Streptophyte	Chlorophytes		
	Cyan	Porp	Guil	Odon		Neph	Pedi	Chlo
1	●				●		●	
2		●	●					
3	●	●	●	●	●		●	
4	●							
5			●		●			
6	●	●	●					
7	●	●	●	●	●			
8	●	●	●		●		●	●
9	●					●		
10						●		
11					●		●	
12					●	●	●	●
13a						●		
13b						●	●	
13c						●	●	●
13d					●	●	●	●
14					●	●		
15						●		
16a						●		●
16b						●	●	●
17					●	●		

Filled circle denotes the presence of a gene cluster: 1, *rbcl-atpB-E*; 2, *chlL-R(ucv)-V(uac)*; 3, *rpoB-C1-C2-rps2-atpH-H-F-A*; 4, *chlB-psaA-B*; 5, *psaA-B-rps14*; 6, *psal-psbJ-L-F-E*; 7, *ycf9-G(ccc)*; 8, *psaJ-P(ugg)*; 9, *ftsW-N(guu)*; 10, *ftsI-psbA*; 11, *clpP-psbB-T-N-H-petB-D-rps9-rpoA-rps11-rpl36-infA-rps8-rpl5-14-16-rps3-rpl22-rps19-rpl2-23*; 12, *psaJ-P(ugg)-W(cca)*; 13a, *rps12-7-tufA-rpl19-ycf4-cemA-petA-L-G*; 13b, *rps12-7-tufA-rpl19-ycf4-cemA*; 13c, *rps12-7-tufA-rpl19-ycf4*; 13d, *petL-G*; 14, *psaC-ndhD*; 15, *ftsW-N(guu)-ccsA-chlL-N*; 16a, *rpl32-cysT-ycf1*; 16b, *cysT-ycf1*; 17, *rps-ll(gau)-A(ugc)-rrf-rf-R(acg)*. Abbreviations of genus names and references for corresponding cpDNAs: Cyan, *Cyanophora*²⁶; Porp, *Porphyra*²⁶; Guil, *Guillardia*²⁷; Odon, *Odontella*²⁸; Meso, *Mesostigma* (this study); Marc, *Marchantia*²⁹; Neph, *Nephroselmis*²⁰; Pedi, *Pedinomonas* (our unpublished data); Chlo, *Chlorella*³⁰.

only in chlorophytes. Multilayered structures are found in chlorophytes and streptophytes, and appear to be an ancestral character of algae, as similar structures have been described in other algal groups⁶.

Methods

DNA sequencing

Chloroplast DNA from *Mesostigma viride* (NIES-296) was isolated from total cellular DNA as an AT-rich fraction by CsCl-bisbenzimidazole isopycnic centrifugation²⁰. This DNA preparation was sheared by nebulization, and 1,500–3,000-bp fragments were recovered by electroelution after agarose gel electrophoresis. These fragments were treated with *Escherichia coli* Klenow fragment and T7 DNA polymerase, and cloned into the *Sma*I site of Bluescript II KS+. After hybridization of the clones with the original DNA used for cloning, DNA templates from positive clones were prepared with the QIAprep 8 Miniprep kit (Qiagen). Nucleotide sequences were determined with the PRISM dye terminator cycle sequencing kit (Applied Biosystems) on a DNA sequencer (model 373; Applied Biosystems) using T3 and T7 primers. Sequences were assembled and analysed as described²⁰. Short genomic regions not represented in the clones analysed were sequenced from PCR-amplified fragments.

Phylogenetic analysis

Genome sequences were retrieved from GenBank. *Pedinomonas* cpDNA sequences are from our unpublished data. Individual protein and rRNA gene sequences were aligned with CLUSTALW 1.74 (ref. 21), alignments were concatenated, and ambiguously aligned regions containing gaps were excluded. The alignments and data sets are available in Supplementary Information. The program packages MOLPHY 2.3b3 (ref. 16), PHYLIP 3.573c²², PUZZLE 4.0.2²³ and SPLITSTREE 2.4²⁴ were used for phylogenetic analyses. Symmetric distance matrices were computed with PUZZLE and PROTDIST²², whereas Logdet distances were calculated with SPLITSTREE. Distance trees were constructed with NEIGHBOR²² and/or FITCH²², maximum-parsimony trees were obtained with PROTPARS²², and quartet-puzzling trees were generated with PUZZLE. The robustness of distance and maximum-parsimony trees was assessed by bootstrap percentages after 100 replications. In the case of quartet-puzzling trees, reliability percentages of the occurrence of the nodes were estimated after 10,000 puzzling steps. Maximum-likelihood analyses of protein and DNA sequences were carried out with PROTML¹⁶ and NUCML¹⁶, respectively, and local bootstrap probability was estimated by resampling of the estimated log likelihood¹⁶.

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Supplementary information is available on Nature's World-Wide Web site (<http://www.nature.com>) or as paper copy from the London editorial office of Nature.

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Pattern recognition and active vision in chickens

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Recognition of objects or environmental landmarks is problematic because appearance can vary widely depending on illumination, viewing distance, angle of view and so on¹. Storing a separate image or 'template' for every possible view requires vast numbers to be stored and scanned, has a high probability of recognition error and appears not to be the solution adopted by primates^{2,3}. However, some invertebrate template matching systems can achieve recognition by 'active vision' in which the animal's own behaviour is used to achieve a fit between template and object⁴, for example by repeatedly following a set path^{5–7}. Recognition is thus limited to views from the set path but achieved with a minimal number of templates. Here we report the first evidence of similar active vision in a bird, in the form of locomotion and individually distinct head movements that give the eyes a similar series of views on different occasions. The hens' ability to recognize objects is also found to decrease when their normal paths are altered.

When leaving a nest or newly discovered food source, bees and wasps use special orientation flights and then attempt to recapitulate the same set of views on subsequent occasions by following similar flight paths^{5–7}. The functional significance of such active vision⁴ is that by learning only a limited set of views of an object, the task of recognizing it is greatly simplified, although at the cost of recognition failure if the object is viewed from an unfamiliar

viewpoint. So far, no clear evidence for the importance of active vision in visual recognition has been documented for vertebrates⁸. Here we report the first evidence, to our knowledge, that object recognition in a bird also involves recapitulating similar views of objects on different occasions by repeated patterns of locomotion and head movements, and a failure of recognition from other viewpoints. We used frame-by-frame video analysis of the body and head movements of six freely moving hens discriminating between two stimulus objects for a food reward under three sets of conditions: (1) when the absolute position of the objects was varied from trial to trial but the birds nevertheless altered their behaviour to give themselves similar viewpoints; (2) when the appearance of the objects was altered slightly and the birds spent longer fixating them from their preferred viewing points; and (3) when a barrier prevented the hens from taking their usual path to the objects.

As stimuli, we used white blocks of wood with three-dimensional shiny ornaments (wing nuts, paper clips, bolts or nuts) glued onto the surface. The blocks were placed in front of two low walls, one of which hid a dish of food. The six hens had to learn which surface ornaments indicated the wall with food behind it, the position of the food (left or right) being varied from trial to trial on a pseudo-random (Gellerman) schedule. For condition (1), eight trials from each of six hens were filmed with an overhead video camera running at 25 frames per second. Videotapes were subsequently analysed by taking *x* and *y* coordinates for mid-head, beak and (front middle) object position for each frame, and then calculating the distance and head angle to both positive and negative stimulus objects. For condition (2), we interspersed a further set of 12 normal trials with 8 probe trials in which these familiar objects were substituted by similar ones in which the surface ornaments on either the front face or the side faces had been removed. For condition (3), we inserted a long barrier (120 cm) between the two stimulus objects so that the birds could not follow their usual path; in eight trials they were forced to make a choice at a much larger distance (120 cm) than normal.

All six hens developed fixed patterns of going round the objects to get at the food behind the relevant wall, regardless of whether the positive object was to the left or the right of the room. In the last 30 trials with training objects (mean was 85% correct), 3 hens always moved around the right-hand side of the stimulus object as they approached it, keeping it on their left in 100% of the trials; 2 hens moved around to the left side, leaving it on their right in 100% of the trials; and 1 hen kept the positive object on its left in over 90% of the trials (number of hens always keeping stimulus object on the same side on at least 90% of trials was 6/6; $P = 0.016$, binomial test). Even more strikingly, each hen showed her own individually distinct pattern of head movements that were very similar from trial to trial but often distinct between hens (Fig. 1a, b). Two birds used predominantly the left eye, two used the right eye, and two switched from one to the other in a predictable sequence. A 'fixation' was defined as a sequence of two or more frames (>80 ms) in which both eye position and head angle were constant^{9,10}. All six hens fixated at individually distinct distances from the stimulus (Fig. 2), with a particular tendency to fixate at around 19–22 cm, a distance previously found to have significance in social interactions¹¹. However, they showed no such consistency for the absolute position in space at which the fixations occurred (number of hens showing fixations at the same distance in at least 5/8 trials was 6/6, $P = 0.016$; number of hens showing fixations at the same head angle in 5 or more trials was 6/6, $P = 0.016$; and number of hens showing fixations at the same *x* coordinate in 5 or more trials was 1/6 and fixations at same *y* coordinate was 0/6). Their view of a stimulus was thus consistently from the same distance, with the same eye and the same head angle, but they did not fixate the stimuli from particular angles. When stimulus objects had ornaments removed from one or more of their faces, the hens showed an increase in the duration of

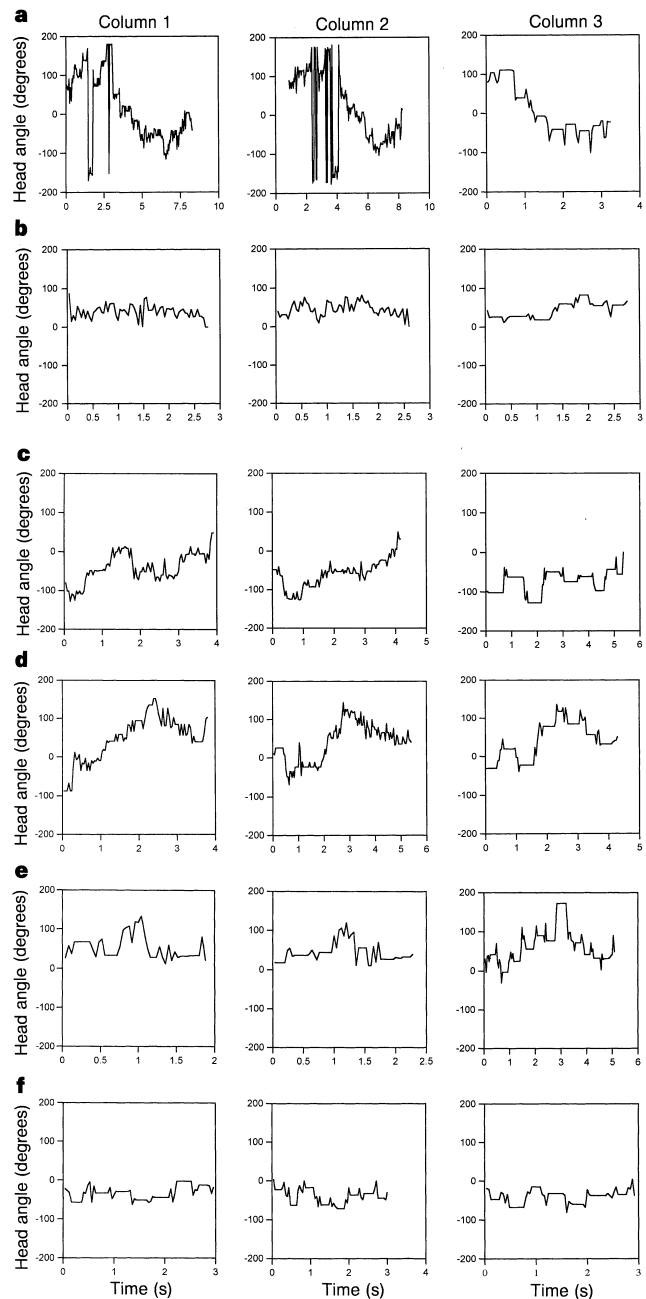


Figure 1 Examples from each of six hens of the head movements on approach to stimulus objects. Movements were recorded every 1/25 second from videotapes. Positive head-angle values indicate that a bird's left eye was pointing towards the positive object; negative angles indicate the right eye. Rows **a–f** indicate hens 1–6, respectively. Columns 1 and 2 show two examples of behaviour from each bird during trials with 'normal' stimuli seen throughout their training. Time 0 is the time at which the bird's head appeared in the video frame approximately 60 cm from the stimuli. Note the striking similarity of the two records for each bird. Only two records per bird are shown for illustration but statistical analyses were done using eight records for each bird. Each sequence was described by six parameters: intercept, linear, quadratic and cubic regression coefficients and a one-lag autocorrelation coefficient to account for serial dependence in the data. We used the transformed head angle ($\log(\text{angle} + 180)$), as the response variable and obtained the estimates of the six coefficients and their standard errors using a linear mixed-effects model. For the mean head angle (indicating which eye was used), there were significant individual differences between hen 3 and the rest, and between hens 2 and 6 ($P < 0.001$). Column 3 shows, for each individual hen, an example of the head movements when the surface ornaments on the front face of the block were removed. There was an increase in duration of 'fixations' (sequences of two or more video frames in which the eye and head angle remained the same). These appear as plateaux on the graph.

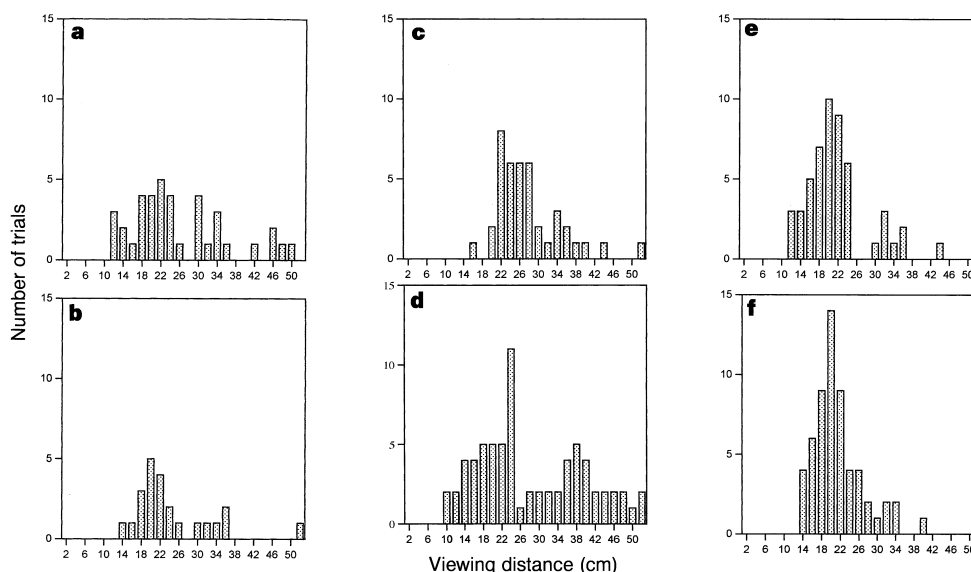


Figure 2 Frequency distribution of fixations at different distances. Shown are fixations (sequences in which the hen's eye position and head angle remained the same for at least 80 ms) that were repeated in at least 5 of 8 trials for the 6 hens. The graphs show that

each hen had individually distinct preferred viewing distances, but that all individuals tended to include 19–22 cm as one of these distances.

fixations (shown by the plateaux in Fig. 1c) from a median of 110 ms in training trials to 310 ms ($P < 0.01$, $n = 6$, $P < 0.01$, Wilcoxon Matched Pairs Test). However, the hens did not consistently use one eye or the other to fixate the changed objects. Rather, they fixated with the eye that they were using at that point in their sequence of head movements (Fig. 1c). Hens using solely the left eye (hens 2 and 5) fixated with the left eye, hens using mainly the right eye (hens 3 and 6) fixated with the right eye, and hens that switched from right to left (hen 4) or left to right (hen 1) fixated with both in sequence. With the long barrier that prevented the birds from taking their normal path, their ability to discriminate fell significantly. All six hens showed lower discrimination with the long barrier (median was 50%) than with the short barrier (median was 90%; $P < 0.01$, $n = 6$, Wilcoxon Matched Pairs Test). All six hens showed significant discrimination of over 70% with the short barrier; only one showed discrimination of over 70% with the long barrier).

These results show that the eyes of an individual hen followed paths through time: an individual hen always went around the same side of an object, exhibited a similar pattern of head movements as it approached an object, and fixated at particular distances, viewing with the same eye and the same head angle on different trials. The visual significance of these individually distinct paths was confirmed by the finding that if the object had an unexpected appearance, the birds increased the amount of time fixating and then moved closer to look again, using the same eye and the same fixation distances as they had previously. Although both eye and head movements stabilize gaze in birds^{9,10}, eye movements are small (maximum 15°)¹²; similarity of head angle thus indicates similarity of eye angle within these limits¹³. Although the hens may have been looking with the same part of the eye, the eyes themselves did not follow the same path through space on different trials, so the hens did not fixate from particular viewpoints, as reported for bees and wasps^{5–7}. However, the stimuli used here were not complex landmarks but decorated blocks that could be recognized by simple key features rather than more complex, angle-dependent 'snapshots'⁵. The results are consistent with the idea that birds use active vision for object recognition, in the form of precisely repeated locomotory and head movements that allow them to recapitulate views of objects from the same distance and with the same part of the eye on successive occasions. □

Methods

Six ISA Brown hens aged between 30 and 50 weeks were individually trained and tested in a room $1.8 \times 3.0 \times 2.0$ m high. At one end of the room was a white wooden starting box ($400 \times 430 \times 480$ mm high), which had both a wooden and a clear perspex door. At the other end of the room, 1.8 m away, were two concrete walls ($200 \times 100 \times 400$ mm high) and 800 mm apart. A barrier (710 cm high) down the mid-line of the room extended 5 cm in front of the stimulus objects and separated the two walls. Which wall hid food was varied from trial to trial, but was always indicated by the presence in front of the wall of a white rectangular block of wood ($75 \times 75 \times 200$ cm high) which had 3 small shiny ornaments glued onto each face. (These were either 3×2 -cm wing nuts one above the other on the front face and 3×2.75 -cm bolts on each of the side faces or 3×2.5 -cm safety pins on the front face and 3×1 -cm bolts on each side face). Food (a mixture of grain and commercial layers mash) was placed in a small dish behind one of the walls. Each wall had cardboard baffles behind it so that an approaching hen could not see whether any food was present until she had gone around the back of it. The front of each brick was covered with bright red card to attract the attention of the birds, and in front of this card, the white wooden blocks were placed on stands so as to be level with the birds' eyes as they approached.

Before a training session, a hen would be taken out of the home pen and confined for one hour in a small cage with water but no food and within sight of its home group of seven other hens. This short period of food deprivation was necessary to motivate the birds to run the trials, and the confinement in a separate cage was so that food did not have to be taken away from all the birds in the home group.

The six hens were first trained to come out of the starting box and go behind a wall (varied between being the one on the left and the one on the right in a Gellerman sequence to avoid the birds developing position preferences) to find food. They were given 8–10 trials per day. For each trial, a hen was confined in the starting box with both doors down. The wooden door was then raised, giving the hen a view of the walls and their associated objects. After 20 seconds, the perspex door was also raised and the hen was free to walk out. If she went behind the wall with the positive stimulus in front of it, she was allowed to feed for 10 seconds and was then picked up by hand and returned to the starting box. For each trial, the hen's choice and which direction she went round the wall to look for food was recorded.

For pre-training, the relevant wall had the positive stimulus in front of it and the other wall had nothing. Once the hens had learned this task, they were presented with both the positive and the negative stimulus and training was continued until the hens had achieved a criterion of 3 successive days with 8 or more of the 10 daily trials correct. This occurred for all hens within between 5 and 10 days. Training was then continued for approximately 10 more days before video recordings were made.

An overhead CCTV camera was positioned 2 m above the floor in the middle of the room and behaviour recorded using a Panasonic VHS recorder. The resulting video tapes were played into an AV Power Mac and analysed frame-by-frame (25 frames per second). For each frame, the x and y coordinates of the position of the middle of the bird's head (between the eyes), its beak tip and the position of the front centre of the positive and negative stimulus objects were recorded with a mouse pointer. These coordinates were then used to calculate the distance from the birds' eye to the stimulus objects and the head angle (the angle between a line drawn from the middle of the bird's head to its beak tip and a line drawn from the head to the object).

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Altered brain response to verbal learning following sleep deprivation

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The effects of sleep deprivation on the neural substrates of cognition are poorly understood. Here we used functional magnetic resonance imaging to measure the effects of 35 hours of sleep deprivation on cerebral activation during verbal learning in normal young volunteers. On the basis of a previous hypothesis¹, we predicted that the prefrontal cortex (PFC) would be less responsive to cognitive demands following sleep deprivation. Contrary to our expectations, however, the PFC was more responsive after one night of sleep deprivation than after normal sleep. Increased subjective sleepiness in sleep-deprived subjects correlated significantly with activation of the PFC. The temporal lobe was activated after normal sleep but not after sleep deprivation; in contrast, the parietal lobes were not activated after normal sleep but were activated after sleep deprivation. Although sleep deprivation significantly impaired free recall compared with the rested state, better free recall in sleep-deprived subjects was associated with greater parietal lobe activation. These findings show that there are dynamic, compensatory changes in cerebral activation during verbal learning after sleep deprivation and implicate the PFC and parietal lobes in this compensation.

Being deprived of sleep for one night impairs performance on many cognitive tasks^{2–6}. Verbal learning is a critical cognitive function whose susceptibility to the detrimental effects of sleep deprivation (SD) has been particularly well replicated^{7–9}. Decreases in specific cognitive functions after SD may be associated with impairments in the cerebral systems that form the neural substrates of these functions¹. In particular, SD has been reported to impair performance on cognitive tasks, including verbal learning tasks, that are putatively dependent upon PFC involvement^{4–6}. These observations led us to propose that some cerebral systems, particularly the PFC, would be less activated by cognitive tasks in sleep-deprived than in rested subjects. To test this proposal directly requires the use of noninvasive techniques that measure localized cerebral function. To our knowledge, only four reports using functional brain-imaging methods have described the effects of SD on cognitive performance^{10–13}. Although none of these studies investigated the effects of SD on verbal learning, two of them reported reduced cerebral metabolic rate in the PFC following SD^{10,12}.

We measured localized cerebral activation using the blood oxygen level-dependent (BOLD) functional magnetic resonance imaging (fMRI) method during performance on a verbal learning task in 13 normal young volunteers after a normal night of sleep (the rested state) and after 34.7 ± 1.2 hours without sleep (the SD state). For each state, the cognitive task alternated between a baseline condition (determining whether a list of nouns was in upper or lower case) and an experimental condition (memorizing a list of nouns)¹⁴. During task performance, BOLD functional images were acquired at each of 20 sagittally oriented slices covering the whole brain. Using high-resolution anatomical images, we identified regions of significant activation during each state separately and regions that were significantly more activated during one state than the other. Significant activation was determined using a cluster threshold method to protect against type I errors¹⁵.

Subjects performed significantly less well on free recall when they were sleep-deprived (4.7 ± 4 words after normal sleep versus 2.8 ± 2 words after SD, $P < 0.05$), but showed no significant change in recognition memory (discriminability index, $d' = 2.5 ± 1$ versus $2.0 ± 1$, $P > 0.05$). Subjective levels of sleepiness on the Stanford sleepiness scale (SSS)¹⁶ increased ($2.3 ± 0.8$ versus $4.4 ± 1.4$, $P < 0.001$) and levels of concentration decreased (this and subsequent subjective measures used a 5-point Lickert scale; $4.5 ± 0.7$ versus $3.5 ± 1.1$, $P < 0.007$). Subjective estimates of effort ($4.0 ± 1.2$

Table 1 Regions of significant brain activation following normal sleep and sleep-deprived nights

Brain regions	Normal sleep		Brain regions	Sleep deprivation	
	Talairach Coordinates	Brodman Areas		Talairach Coordinates	Brodman Areas
L. MFG	33L, 58A, 6S	10*	L. SFG	18L, 30A, 45S	8
L. MFG/IFG	47L, 24A, 11S	8,9/45,47†‡	L. MFG/OFG	29L, 54A, 4S	10*§
L. OFG	12L, 54A, 14I	10§	L. MFG	52L, 20A, 31S	8/9/46†
L. AC	4L, 31A, 13S	32, 24	L. IFG	53L, 24A, 1I	47‡
L. AC	5L, 21A, 10I	32, 35	L. AC	10L, 24A, 37S	32
L. PMA & SMA	15L, 11A, 61S	6	R. MOG	25R, 92P, 13S	18, 19
	41L, 5A, 25S	6			
	47L, 3A, 42S	6			
	25L, 18A, 53S	6			
L. TP	34L, 4A, 18I	38			
L. MTG	56L, 22P, 5I	21			
L. SOG	43L, 72P, 37S	19			

Each entry represents a significant cluster of activation. Clusters that physically overlap between nights are denoted with identical symbols (*, †, ‡ or §). Premotor area (PMA) and supplementary motor area (SMA) activation after the normal night of sleep included four discrete clusters. The anterior cingulate (AC) gyrus activation after the SD night was slightly dorsal (superior) to that observed after the normal night of sleep. SFG, MFG, IFG and OFG: superior, middle, inferior and orbital frontal gyri, respectively; MTG: middle temporal gyrus; SOG and MOG: superior and middle occipital gyri, respectively. L, left hemisphere; R, right hemisphere. The magnitude of activation of every pixel of each cluster was significant at a minimum t -value of 2.18, degrees of freedom 12.

versus 4.1 ± 1.0) and of task difficulty (1.2 ± 0.4 versus 1.6 ± 0.8) did not change significantly between the rested and sleep-deprived states.

In the rested state, the left PFC, premotor area and temporal lobe were activated during the verbal learning task (Table 1). Contrary to our hypothesis, discrete regions of the PFC were more activated during verbal learning following SD than following normal sleep. The temporal lobes were significantly more activated during the rested state than during the SD state (Fig. 1a). Additionally, the bilateral parietal lobes (Fig. 1b) and two additional frontal lobe regions (left middle frontal gyrus and right inferior frontal gyrus) were more activated after SD than in the rested condition. These regions are involved in tasks with high working memory or cognitive loads^{17–20} and also include the areas hypothesized to be the site of the short-term memory store^{20,21}.

Simple repetition of the verbal learning task might alter BOLD response through practice effects or habituation. To determine whether brain areas that showed altered BOLD response following sleep deprivation also showed altered BOLD response induced by repetition of the verbal learning task, we performed a paired *t*-test comparing the first and second nights of activation. There was no overlap between areas showing sleep deprivation effects and areas showing repetition effects.

To explore further the relationships among cerebral activation, SD and cognitive performance, we regressed cerebral activation during the SD night onto participants' subjective levels of sleepiness and onto their free recall performances. Increased sleepiness was significantly correlated with activation in two bilateral PFC regions (left inferior frontal gyrus at BA47 and right superior/middle frontal gyri at BA10) and one in the right cerebellum. Furthermore, better free recall after SD was associated with greater activation in three regions: within the bilateral parietal lobes and one each in the right temporal gyrus and the left supplementary motor area. Subjective ratings of effort and of task difficulty obtained during SD did not significantly correlate with activation of the brain sites that were more responsive during SD.

Several authors have argued that some aspect of cerebral dysfunction following SD impairs cognitive performance compared with the rested state^{1,3,5–6}. This study partially supports this argument (there is loss of left temporal lobe activation after SD), and is consistent with a preliminary report suggesting that SD alters the overall pattern of cerebral activation during task performance¹³. Our data also indicate that the brain may be able to compensate for the effects of SD while maintaining at least partially intact performance. Following SD, performance is often initially intact and then declines with increasing time-on-task, suggesting that individuals can initially compensate for the effects of SD. Here, SD led to increased BOLD signals during verbal learning in bilateral working memory regions of both the frontal and parietal lobes. Thus, we propose that these regions may represent the neurophysiological substrate of the initial compensation for SD. Such a compensatory mechanism has been proposed for cognitive performance during other types of stressor²². Given the relatively short duration of our task, we may have only measured that period of time when the brain is able to compensate for SD and maintain relatively intact performance. It is possible that had we used a longer task, we might have observed a smaller compensatory brain response.

Following SD, activation within PFC was significantly correlated with sleepiness, whereas activation within the parietal lobes was related to preservation of near-normal verbal learning. The significant relationship between increased prefrontal cortical response to verbal learning and increased sleepiness is of particular interest for two reasons. First, during non-rapid eye movement (nonREM) sleep, increased delta frequency in the electroencephalogram (the frequency associated with deep slow-wave sleep) occurs earlier in the prefrontal cortex than in the rest of the cortex, especially after SD²³. Second, levels of adenosine in the basal forebrain rise mono-

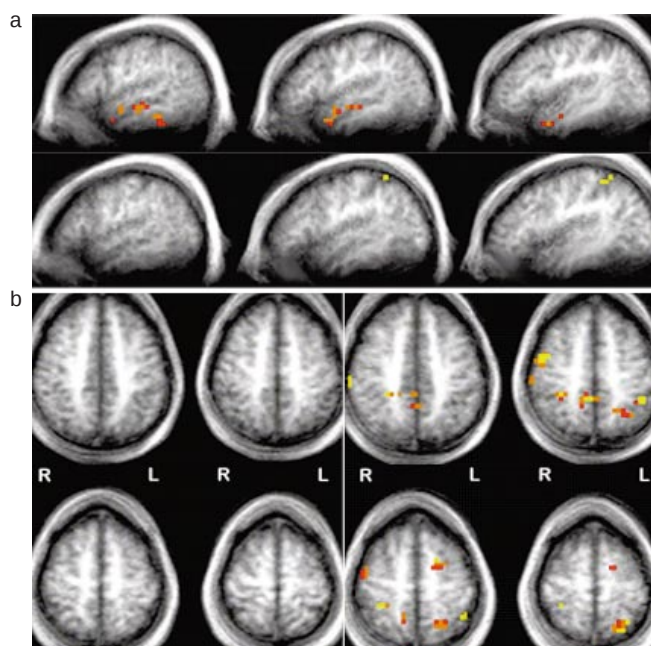


Figure 1 Within-subject significant *t*-tests for mean differences in activation between rested and SD nights. Red, smallest differences; yellow, largest; $n = 13$. **a**, Both panels show left hemisphere sagittal slices from lateral to medial at 51, 47 and 43 mm from midline (left to right). Top: areas showing increased activation after normal sleep. Areas not shown include the anterior cingulate, left premotor area and medial and left cerebellum. Bottom: areas showing increased activation after SD. **b**, Both panels show axial slices above the inter-commissural line (clockwise from upper left: 43, 48, 58 and 53 mm). Left: no significant activation in any area in these slices after normal sleep. Right: increased activation after SD. Additional areas include left middle frontal gyrus, right inferior frontal gyrus, right inferior temporal gyrus and superior occipital gyrus.

tonically with increasing time spent awake and might be responsible for the induction and maintenance of nonREM sleep²⁴. One might speculate, then, that the increased prefrontal response to verbal learning after SD reflects compensation for the direct or indirect effects of adenosine, or of other endogenous sleep-promoting substances in the basal forebrain. Under this hypothesis, the increased prefrontal cortex response to verbal learning after SD would represent compensation for the increased homeostatic drive for sleep. The increased parietal activation seen after SD, on the other hand, may underlie partially successful behavioural adaptation to SD during verbal learning. The compensatory response of the parietal lobe to SD might be restricted to cognitive functions that do not activate the parietal lobes during the rested state. For example, serial arithmetic activates the parietal lobes when subjects are rested, and SD impairs serial arithmetic and is associated with a diminished parietal BOLD response to arithmetic²⁵.

Although changes in effort might affect neural activation in brain regions associated with working memory, increased effort is unlikely to explain the increased PFC and parietal response to verbal learning following SD. Neither subjective levels of effort nor perceived task difficulty changed with SD or correlated significantly with localized brain activation following SD.

Behavioural compensation for SD was not complete, and some of the changes in cerebral activation that followed SD may have contributed to poorer recall performance. In particular, the reduced response of the left temporal lobe to verbal learning following SD might have been associated with lowered recall scores. Decreased temporal lobe activity is associated with poor verbal learning in

pathological conditions, such as Alzheimer's disease, where reduced perfusion of the temporal lobe predicts the rate of decline of verbal memory²⁶.

In summary, the neural mechanisms mediating cognitive performance after SD are complex and dynamic, and differ, in part, from those employed in the rested state. The effects of SD on cognitive performance and functional cortical activation illustrate the brain's plasticity and, at times, its attempts to compensate for the failure of normal neural systems during the execution of specific tasks. In the case of verbal learning, haemodynamic responses increased following SD in both the frontal lobes (perhaps responding to or reflecting sleepiness) and in the parietal lobes (perhaps assisting with the achievement of near-normal free recall) despite an apparent loss of temporal lobe involvement. In contrast, in the case of serial subtraction²⁵, haemodynamic responses were significantly reduced after SD in the frontal and parietal lobes as performance faltered. These observations indicate that the effects of SD on cognitive performance and related patterns of cerebral activation may depend in part on task-specific demands. □

Methods

Subjects and behavioural conditions

Thirteen normal healthy subjects (mean age 27.2 years, range 21–35 years; mean education 16.5 years, range 14–18 years) participated after providing written informed consent. All subjects were screened using a medical and psychiatric history, physical and laboratory examinations and an overnight sleep laboratory evaluation to establish that they had relatively normal sleep patterns. During SD, subjects were monitored in a hospital from 22:00 until the time of the scan, around 16:30–18:00 the next day, and were not allowed stimulants of any kind. The order of the rested and SD conditions was counterbalanced. Subjects performed four separate cognitive tasks while undergoing fMRI scans in both the rested and SD states. The study of serial subtraction has been reported elsewhere²⁵. The present study concerns verbal learning, as described below. The behavioural trials alternated between four experimental and five baseline blocks, starting and ending with a baseline block (each block, 40 s; total trial, 360 s). Five words were presented during each block; subjects were told to not memorize the baseline words, but to determine whether they were in all upper-case or all lower-case letters. Subjects were instructed to actively memorize the experimental words for later testing. Free recall and recognition were tested outside the scanner about 10 min after the end of the functional scans. We analysed free recall data using a paired-samples *t*-test, and recognition memory performance using the discriminability index *d'*. After completion of the memory tests, subjects rated, on a series of 5-point Likert scales, their subjective levels of concentration, effort and the difficulty of the task. In addition, participants rated subjective sleepiness before and after the scanner procedures¹⁶.

fMRI procedures and data analysis

All images were acquired with a 1.5T GE Signa scanner equipped with an inserted three-axis balanced torque head gradient coil designed for rapid switching²⁷. Whole-brain manual shimming further enhanced the signal-to-noise ratio. Functional images consisted of 90 repetitions of echo planar images (TR, 4,000 ms; TE, 40 ms; FOV, 24 cm; image matrix, 64 × 64; in-plane size, 3.75 mm²; slice thickness, 6 mm; no interslice gap) acquired continuously in an interleaved fashion. For anatomical images, we collected 128 1.5-mm contiguous images using the magnetization prepared rapid acquisition gradient echo (MPRAGE) protocol (256 × 256 matrix; FOV, 24 cm; flip angle, 10°).

All analyses were conducted using Analysis of Functional NeuroImages (AFNI) software²⁸. After motion correction, the functional time-course data from each voxel were correlated with a series of 13 reference functions—one seed reference function representing the alternating time course of the behavioural trial and the same reference function shifted in 1-s increments six times both forwards and backwards in time¹⁴. AFNI then used only the reference function that produced the highest correlation with the time-course data. Shifting the reference function takes into account the delays in onset of the haemodynamic response and in the acquisition of the first and final slices²⁹. These correlations were performed within-subject before combining data during group analyses. Before performing group analyses, all individual data sets were transformed into standard atlas³⁰ and spatially smoothed with a Gaussian kernel equal to 3.75 mm full-width-half-maximum. Within-night analyses consisted of performing one-sample *t*-tests on each voxel with a null hypothesis that the contrast between the experimental and control blocks averaged across subjects equaled zero. Between-night analyses consisted of performing paired samples *t*-tests with a null hypothesis that the average signal contrast between the experimental and control blocks on each night was equal. All *t*-tests examined the image intensity derived from the correlation of the time-course data with the reference functions as the dependent variable and used the variance in image intensity across subjects to test for significant BOLD response in each individual voxel. Hypotheses were related to areas significantly more activated by the experimental task than the baseline task; therefore, only positive activation was examined. The regression analysis performed on the SD data used the increase in SSS scores between the normal sleep scan and the SD scan (SD – normal sleep) to control for basal levels of sleepiness. The change in free recall performance from

the normal to the SD night was used to estimate the degree to which SD affected performance (normal sleep – SD: larger values represent greater impairment). Both positive parameter estimates (areas associated with greater performance decrements) and negative parameter estimates (areas associated with more intact performance) were examined for this variable.

The cluster thresholding method to control for type I error associated with multiple statistical tests was as described¹⁵. Based on 10,000 Monte Carlo simulations, we defined a significant activation as a cluster of at least six contiguous voxels, each individually activated at an *a priori* alpha level < 0.025 (1-sided). This resulted in a final per-voxel *P*-value of 0.0005 (1-sided).

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The organizer factors Chordin and Noggin are required for mouse forebrain development

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In mice, there is evidence suggesting that the development of head and trunk structures is organized by distinctly separated cell populations^{1,2}. The head organizer is located in the anterior visceral endoderm (AVE) and the trunk organizer in the node and anterior primitive streak. In amphibians, Spemann's organi-

zer, which is homologous to the node, partially overlaps with anterior endoderm cells expressing homologues of the AVE markers *cerberus*, *Hex* and *Hesx1* (refs 3–6). For mice, this raises the question of whether the AVE and node are independent of each other, as suggested by their anatomical separation, or functionally interdependent as is the case in amphibians^{3–5}. Chordin and Noggin are secreted bone morphogenetic protein (BMP) antagonists^{7,8} expressed in the mouse node, but not in the AVE. Here we show that mice double-homozygous mutants that are for *chordin* and *noggin* display severe defects in the development of the prosencephalon. The results show that BMP antagonists in the node and its derivatives are required for head development.

The signalling activities of BMPs can be antagonized by a diverse group of binding proteins, including Chordin and Noggin^{1,2,7,8}. Mouse *chordin* (*Chd*) messenger RNA is first expressed in the anterior primitive streak and then in the node and axial mesendoderm that derives from it (Fig. 1a), suggesting that *chordin* may play a role in patterning the early embryo. However, targeted inactivation of *chordin* results in stillborn animals that have normal early development and neural induction but display later defects in inner and outer ear development (Fig. 1e and f) and abnormalities in pharyngeal and cardiovascular organization (D.B. *et al.*, manuscript in preparation). At midgastrula, expression of *noggin* (*Nog*)⁹ overlaps with *chordin* (Fig. 1b and c). The *noggin* mutants undergo normal gastrulation and anterior central nervous system (CNS) patterning, although at later stages a number of abnormalities are

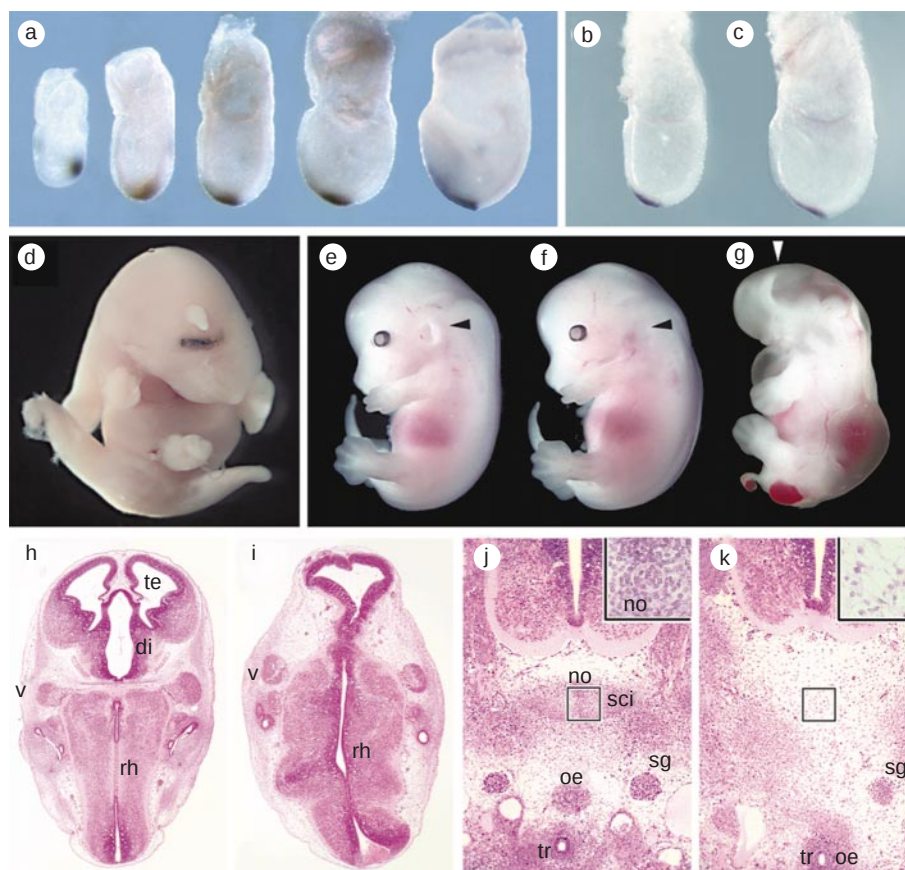


Figure 1 Expression of *chordin* and *noggin* in the node and phenotype of double mutants. **a**, Expression of *chordin* during gastrulation in anterior primitive streak, node and axial mesendoderm. **b** and **c**, Expression of *noggin* at neural plate and head fold stages; expression overlaps with that of *chordin*. We note that *chordin* and *noggin* are not expressed in the AVE. **d**, *Chd*^{-/-}; *Nog*^{-/-} embryo recovered at late gestation, showing single nasal pit (proboscis), cyclopia and agnathia. **e–k**, E12.5 embryos. **e**, Wild-type (wt). **f**, *Chd*^{-/-}; *Nog*^{+/-}. We note the normal head and defective ear (arrowhead). **g**, *Chd*^{-/-}; *Nog*^{-/-} showing extensive anterior deletions of forebrain, eye, nose and facial structures. White arrowhead indicates the rhombic lip (future cerebellum); all external phenotypes

posterior to it are also seen in *noggin* mutants⁹. Of the three double mutants recovered at E12.5 (out of a total of 10 expected, $n = 153$) two had this phenotype, and one retained a small cyclopic eye. **h** and **i**, Coronal sections of the wild-type and double-mutant embryos shown in **e** and **g**; in the mutant the rhombencephalon (rh) is relatively normal, whereas the cerebral hemispheres telencephalon (te) and diencephalon (di) are reduced to a single thin neural vesicle. The fifth (trigeminal) ganglion (v) is indicated. **j** and **k**, Transverse sections of wild-type and double-mutant embryos. We note the absence of notochord (no, see inset) and sclerotome (scl) at this cervical level; the oesophagus (oe) and trachea (tr) form a common duct^{20,21} in the mutant. The sympathetic ganglion (sg) is indicated.

observed in posterior spinal cord and somites⁹. These results contrast with overexpression experiments in *Xenopus*, in which the activities of *chordin* and *noggin* have been shown to dorsalize the embryo and induce anterior neural tissue in animal cap explants^{7,8}. As both *chordin* and *noggin* have similar biochemical activities and overlapping expression domains during gastrulation, we reasoned that they might have redundant activities during mouse gastrulation, which could be revealed by generating compound mutants.

Intercrosses were set up between compound heterozygous mice and no double-homozygous mutants were recovered among the neonates ($n = 320$, 20 expected). We therefore dissected pregnant mothers at progressively earlier stages of development. Two *Chd*^{-/-};*Nog*^{-/-} embryos were found among animals dissected close to term. Both were undergoing resorption, but clearly had holoprosencephaly, with a single nasal pit (proboscis), a cyclopic eye and agnathia (Fig. 1d). These malformations, not observed in either mutant on its own, represent the weakest phenotypes found in *Chd*^{-/-};*Nog*^{-/-} mice and resemble embryos lacking *sonic hedgehog* (*Shh*)¹⁰.

At embryonic day 12.5 (E12.5), double-mutant embryos were recovered with more severe phenotypes resembling aprosencephaly. Anterior to the hindbrain (Fig. 1g, white arrowhead), embryos lacked extensive areas of the forebrain, as well as eyes, nasal placodes and facial structures. Histological sections showed that the cerebral hemispheres (telencephalon) and diencephalon were reduced to a small vesicle of thin neuroepithelium, whereas more posterior brain structures were relatively normal (compare Fig. 1h and i).

In double-mutant embryos dissected at E8.5, the forebrain reduction was clearly evident (Fig. 2a–f). Transcripts of *Six-3*, an anteriormost brain marker¹¹, were almost entirely absent, whereas expression of *Krox-20* in rhombomeres 3 and 5 was present in the hindbrain (Fig. 2a and b). *Engrailed-1* (*En-1*), a marker for mid-brain and anterior hindbrain, was expressed in the double mutants, indicating that the neural defects resided in the prosencephalon (Fig. 2c and d). These deficits were also seen in the anterior neural folds at early somite stages (Fig. 2g–l).

To investigate the onset of the forebrain phenotype and the role of the anterior endoderm¹², we analysed the expression of a number of markers at earlier stages. In double-mutant embryos dissected at E7.5, the expression of the anterior marker *Hesx1/Rpx* (ref. 12) was undetectable both in the endoderm and in the overlying ectoderm that would become the anterior neural plate (Fig. 3a–c). Interestingly, *Hesx1* was expressed normally in the anterior endoderm at early gastrula stage (Fig. 3h and i). Mutational analysis has shown that *Hesx1* is required for normal forebrain development in mice and humans¹³. The lack of *Hesx1* expression is accompanied by an indentation at the anterior border separating the embryonic and extraembryonic regions (Fig. 3b and c, arrowheads). More severe constrictions of this type are seen in mutants that lack head structures, such as *Lim1*, *Hnf3 β* and *Otx2* (refs 14–16) and are caused by defects in the endoderm^{16,17}. The expression domains of *noggin* (Fig. 1b) and *chordin* (Fig. 1a, middle embryo) do not overlap with that of *Hesx1* (Fig. 3a), suggesting that the mutations have effects on the anterior endoderm at a distance. To test whether AVE formation is initiated normally but fails to be maintained, we examined expression of the AVE markers *Hesx1*, *Lim1*, *Cer-like* and *Hnf3 β* (refs 2, 12 and 18) at E6.5. At these early streak stages, all AVE markers tested were expressed normally in double *noggin* and *chordin* homozygous mutants (Fig. 3d, e, h–k and data not shown). This is in agreement with the recent finding that *Wnt3*^{-/-} embryos, in which the entire primitive streak is lacking, still form an AVE (ref. 19). As is the case for *Hesx1*, the expression of *Cer-like* fails to be maintained in the anterior visceral endoderm, as well as in definitive endoderm emerging from the node¹⁸, by the late gastrula stage (Fig. 3f and g). We conclude from these data that *chordin* and *noggin* are not necessary for establishing the AVE but are required for subsequent elaboration of anterior pattern.

Mesodermal development is also affected in double-mutant embryos. This is indicated by the lack of *Shh* (Fig. 2b and d) and *Hnf3 β* (Fig. 2j and l) expression in the rostral midline. At E12.5 the notochord and surrounding sclerotome are absent in cervical regions (insets, Fig. 1j and k). The anterior notochord may initially form and then degenerate, because we observed some E8.5 embryos in which the notochord was present throughout the length of the animal (Fig. 2e', f'). In the endoderm, the anterior pharynx is reduced (not shown), and the trachea does not form, with a common tracheo-oesophageal duct connecting the pharynx to the lungs and stomach (Fig. 1j and k), as has been reported for mutations of the *Shh* pathway^{20,21}. In addition, the absence of Chordin and Noggin also affects left–right asymmetry. Of seven double-mutant embryos (E8.5) in which heart looping could be

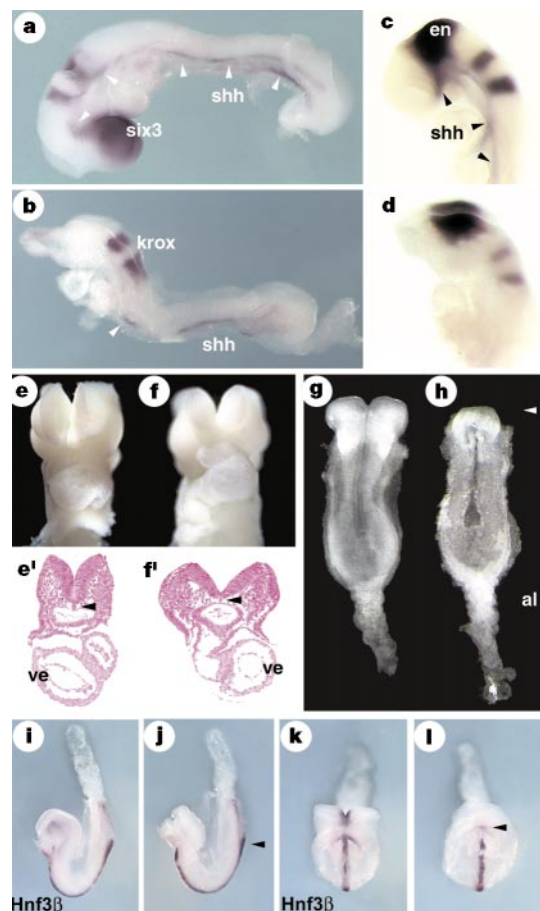


Figure 2 Loss of forebrain in *chordin;noggin* double mutants. **a** and **b**, wild-type and *Chd*^{-/-};*Nog*^{-/-} E8.5 embryos hybridized with *Six-3* and *Krox-20*. We note that forebrain tissue expressing *Six-3* is almost completely missing and that *Krox-20* is expressed in the double mutant. The white arrowhead in **b** marks the level of anteriormost expression of *Shh*. **c** and **d**, wild-type and double-mutant embryos showing that *En-1* and *Krox-20* are expressed in the mutant; anterior *Shh* expression (arrowheads) is observed in the wild type but not in the mutant. **e** and **f**, Slightly earlier embryos showing reduction in anterior neural folds and inverted looping of the heart in the double mutant. **e'** and **f'**, Transverse sections of the embryos in **e** and **f**. The prospective heart ventricle (ve) loops to the right in wild-type and to the left in the double-mutant; the arrowhead indicates the notochord. At E8.5 stage 14 double mutants were recovered out of 18 expected ($n = 267$ embryos genotyped). **g** and **h**, Wild-type and double mutant embryos at early somite stage; the anterior neural folds are defective (arrowhead) and the allantois (al) composed of ventral mesoderm is prominent in this double mutant. **i**, **j**, **k**, **l**, Wild-type (**i** and **k**) and double-mutant (**j** and **l**) E8.0 embryos hybridized with *Hnf3 β* . The same embryos are shown in lateral and frontal view; *Hnf3 β* expression is normal in the node (arrowhead in **j**) and most of the midline, except for the anterior head and foregut pocket region (arrowhead in **l**).

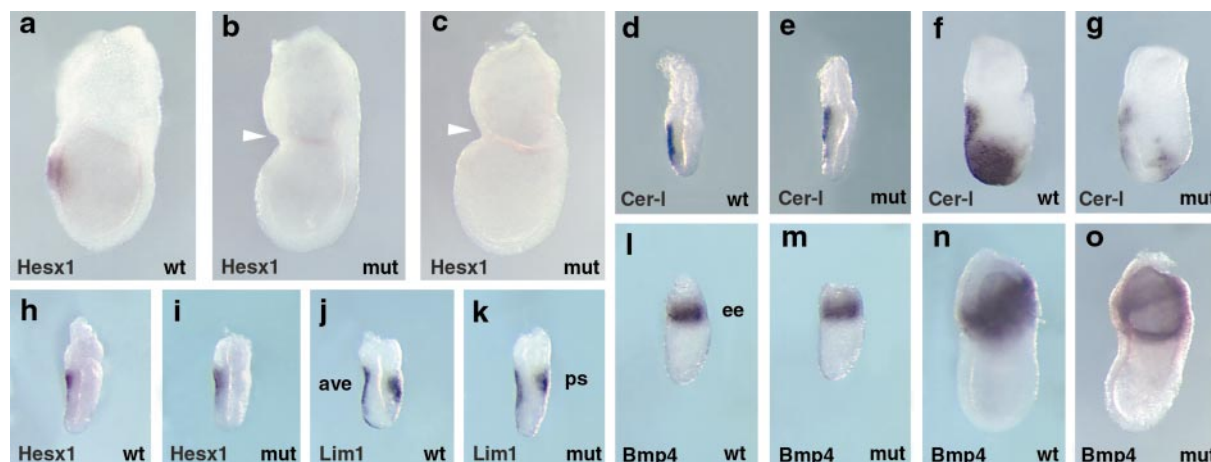


Figure 3 Molecular marker analyses of double *chordin;noggin* mutants during early development. **a**, *In situ* hybridization with *Hesx1* probe at the early neural-plate stage (E7.5) in a wild-type (wt) embryo; the probe stains both the endoderm underlying the future forebrain and the overlying epiblast in which the forebrain is forming. **b** and **c**, Two *Chd*^{-/-}; *Nog*^{-/-} embryos (mut) showing absence of *Hesx1* expression. Note the presence of a mild constriction (arrowhead) at the anterior embryonic–extraembryonic border. **d**, **e** and **h**, **k**, Wild-type and double-mutant embryos hybridized at primitive streak stages with *Hesx1*, *Lim1* and *Cer-like*; anterior visceral endoderm (ave) and primitive streak (ps) are formed normally in the mutants. **f**, **g**, At the late streak stage *Cer-like* expression fails to be maintained in the anterior endoderm of double-mutant embryos. **l**, **m**, Expression of

Bmp-4 in extraembryonic ectoderm (ee) in early/midstreak embryos. **n**, **o**, *Bmp-4* expression at node stage. In both wild-type and mutant embryos expression remains confined to the extraembryonic region. The wild-type embryos shown here may be heterozygous for one of the genes; the mutants were in all cases genotyped as *Chd*^{-/-}; *Nog*^{-/-}. At least two *Chd*^{-/-}; *Nog*^{-/-} embryos were documented for each probe and developmental stage, with the exception of *Hesx1* at early gastrula for which only one double-mutant embryo was recovered. Embryos at these early stages were genotyped by polymerase chain reaction after *in situ* hybridization and photography (*n* = 310); double-homozygous mutant embryos were recovered at mendelian ratios (*n* = 21).

clearly discerned, four had inversions, indicating a randomization of the heart situs (Fig. 2e, f, e' and f'). We conclude that the anti-BMP factors Chordin and Noggin are required for development and maintenance of dorsal mesoderm (notochord, sclerotome, foregut), as well as for left–right patterning. Mutations in either *Hnf3β* or *Shh*, genes downregulated in *Chd*^{-/-}; *Nog*^{-/-} embryos, cause similar mesodermal phenotypes^{17,22}.

In zebrafish, mutations in *chordin* affect patterning of both neurectoderm and mesoderm, causing reduction of neural and of dorsal mesoderm markers^{23,24}. In *chordino* mutants the *Bmp-4* expression domain is expanded into the dorsal region of the gastrula²³. In contrast, this transcriptional regulation is not seen in mice; in *Chd*^{-/-}; *Nog*^{-/-} embryos *Bmp-4* transcripts remain confined to extraembryonic tissues during gastrulation stages (Fig. 3l–o). This observation illustrates differences in the regulatory mechanisms by which mouse and zebrafish gastrulae are patterned. Despite the unchanged *Bmp-4* expression, removal of the antagonists is expected to cause an increase in BMP signalling, because Chordin and Noggin bind BMPs in the extracellular space, which prevents their binding to BMP receptors²⁸. In addition to *Bmp-4*, other BMPs are expressed at neural-plate and head-fold stages. *Bmp-2*, 5 and 7 are expressed in the anterior mesoderm and neurectoderm adjoining the prospective headfolds^{25–27}. *Bmp-7* is also expressed during gastrulation in the node and axial mesoderm, overlapping with the expression domains of *chordin* and *noggin*. Thus, the phenotypes observed in the double mutants may result from increased activity of multiple BMPs. Ectopic application of BMP proteins has been shown to cause holoprosencephaly²⁸, inhibition of cell proliferation and increased cell death in E10.5 forebrain explants²⁹, and decreased expression of *Shh* in the CNS (ref. 26). Although loss of Chordin and Noggin leads to defects in forebrain development, the formation of the midbrain and more posterior CNS is relatively normal; therefore, other regulatory mechanisms must come into play.

As neither *chordin* nor *noggin* are expressed in the visceral endoderm (Fig. 1a–c, ref. 14), this study specifically tests the influence of patterning factors derived from the anterior primitive

streak and node on the development of prospective forebrain. The AVE is initially formed in *Chd*^{-/-}; *Nog*^{-/-} embryos, as shown by the normal expression of *Hesx1*, *Lim1* and *Cer-like* in this region (Fig. 3d, e, h–k). In *Chd*^{-/-}; *Nog*^{-/-} embryos, anterior defects are apparent as early as the start of neurulation (E7.5), when the anterior endodermal expression domain of *Hesx1* underlying the future forebrain is no longer detectable; these antagonists are therefore first required at, or before, this stage (Fig. 3a–c). Two interpretations of the observed phenotypes are possible. First, the node and mesodermal cells emerging from it may have an essential head-inducing activity, and this activity is deficient in *Chd*^{-/-}; *Nog*^{-/-} embryos. Second, anti-BMP signals secreted by the node and its derivatives may be required for the maintenance of anterior patterning initiated by the AVE. In *Xenopus*, expression of *cerberus* and *XHex* in the topological equivalent of the mouse anterior endoderm is maintained by signals from the Spemann organizer, and these can be mimicked by *chordin* or *noggin* mRNA microinjections^{3–5}. Conversely, the expression of *cerberus* in *Xenopus* during gastrulation is decreased by microinjection of *Xoloid* mRNA encoding a protease that inactivates Chordin³⁰ (E. Agius, M. Oelgeschläger and E.M.D.R., unpublished observations). The results presented here demonstrate that the BMP antagonists Chordin and Noggin compensate for each other during early mouse development. When both gene products are removed, antero-posterior (forebrain defects), dorso-ventral (holoprosencephaly in milder phenotypes, notochord and sclerotome defects) and left–right (randomization of heart situs) patterning are affected. Thus, the BMP antagonists Chordin and Noggin secreted by the trunk organizer are required for the proper specification of the three body axes in the mouse embryo. □

Methods

Mutant Mice

The *chordin* mutant allele was generated by inserting stop codons in all three possible reading frames of the signal peptide and by introducing a frameshift immediately after this (D.B. et al., manuscript in preparation). Double homozygotes were obtained by crossing

compound heterozygous *noggin*⁹ and *chordin* parents in a B6SJLF1 background (Jackson Laboratories).

Genotyping

DNA was extracted from extraembryonic membranes of E8.5 and older embryos. For E6.0 to E7.5 embryos the whole animal was digested overnight upon completion of *in situ* analysis and photography of each embryo. Digestion was at 55 °C in 50 mM Tris-HCl, pH 8, 100 mM EDTA, 100 mM NaCl, 1% SDS, 0.5 mg ml⁻¹ Proteinase K. After adding NaCl to 1 M final concentration, the mix was centrifuged for 15 min, the supernatant recovered, and DNA precipitated with an equal volume of isopropanol. The genotype of the specimens was determined by polymerase chain reaction using the following primers: *chordin* primers were a mixture of three oligonucleotides, Chd-F (5'-GAGTTAGGAGG-TGGAGCTCTACAC-3'), Chd-R (5'-GGTAGGAGACAGAGAAGCGTAAACT-3') and Neo-2 (5'-GTTCCACATACACTTCATTCTCAG-3'), which yielded bands of 417 and 285 base pairs (bp) for the wild-type and mutant alleles respectively. *Noggin* primers were a mixture of three oligonucleotides, Nog-1 (5'-GCATGGAGCGCTGCCAGC-3'), Nog-2 (5'-GAGCAGCGAGCGCAGCAGC-3') and β-Gal (5'-AAGGGCGATCGGTGC-GGGCC-3'), which yielded bands of 211 and 160 bp for the wild-type and mutant alleles respectively.

In situ hybridization

Whole-mount *in situ* hybridization was performed as described¹⁸. The *chordin* probe used was a subclone spanning nucleotides 145–2016 of the full-length mouse *chordin* complementary DNA (accession number AF096276), linearized with *NotI* and transcribed with T3 RNA polymerase. The *Krox20* probe was linearized with *Bam*H1 and transcribed with T3 RNA polymerase. *En-1*, *Hnf3β* and *Bmp-4* were linearized with *Clai*, *Asp*700 and *Bam*H1 respectively, and transcribed with T7 RNA polymerase. Other antisense probes used were: *noggin* (ref. 9), *Shh* (ref. 9), *Six3* (ref. 11), *Hesx1* (ref. 12), *Lim1* (ref. 14) and *Cer-1* (ref. 18).

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Maintenance of functional equivalence during paralogous Hox gene evolution

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Biological diversity is driven mainly by gene duplication followed by mutation and selection. This divergence in either regulatory or protein-coding sequences can result in quite different biological functions for even closely related genes. This concept is exemplified by the mammalian Hox gene complex, a group of 39 genes which are located on 4 linkage groups, dispersed on 4 chromosomes^{1–4}. The evolution of this complex began with amplification in *cis* of a primordial Hox gene to produce 13 members, followed by duplications in *trans* of much of the entire unit. As a consequence, Hox genes that occupy the same relative position along the 5' to 3' chromosomal coordinate (*trans*-paralogous genes) share more similarity in sequence and expression pattern than do adjacent Hox genes on the same chromosome. Studies in mice indicate that although individual family members may have unique biological roles, they also share overlapping functions with their paralogues^{5–12}. Here we show that the proteins encoded by the paralogous genes, *Hoxa3* and *Hoxd3*, can carry out identical biological functions, and that the different roles attributed to these genes are the result of quantitative modulations in gene expression.

The paralogous genes *Hoxa3*, *Hoxb3* and *Hoxd3* show virtually identical expression patterns of messenger RNA and share, in a pairwise comparison, ~50% identity in protein-coding sequences^{13–17}. Nevertheless, mice lacking either a functional *Hoxa3* or *Hoxd3* gene show no obvious overlap in phenotype: the former have deficiencies in pharyngeal tissues derived from mesenchymal neural crest¹⁸, the latter in somitic, mesodermally derived tissues of the axial skeleton¹⁹. Although the uniqueness of the single-mutant phenotypes implies a different qualitative role for each gene product, the analysis of animals carrying different combinations of mutant alleles of the *Hox3* paralogues suggests that there is also functional overlap between the three genes, and that the important parameters that mediate these interactions may be quantitative in nature^{5,17,20–22}. For example, in animals that are

homozygous for null alleles of *Hoxd3*, the defect in the formation of the atlas is primarily restricted to the anterior arch¹⁹. The severity of this phenotype is increased quantitatively by a serial reduction in the number of functional copies of either the *Hoxa3* or the *Hoxb3* genes. In the *Hoxd3*^{-/-} background, absence of one copy of either paralogue reduces the size of the entire atlas by ~50%; absence of any two copies, either *Hoxa3*^{-/-} or *Hoxb3*^{-/-}, or *Hoxa3*^{+/-};*Hoxb3*^{+/-}, eliminates the entire atlas^{5,17}.

To examine directly the nature of functional overlap^{8,23,24} within the *Hox3* family, we have exchanged reciprocally, in the genome of mice, the protein-coding portions of the *Hoxa3* and *Hoxd3* genes

(Fig. 1a). Thus, we have generated mice that lack any HoxA3 protein, but instead express the HoxD3 protein from both the *Hoxa3* and *Hoxd3* loci (Fig. 1b), as well as mice that lack HoxD3 protein but express HoxA3 from both loci. The alleles used in this study are shown in Fig. 1c and include the two wild-type alleles, *Hoxa3*⁺ and *Hoxd3*⁺; the null alleles, *Hoxa3*^{null} and *Hoxd3*^{null}; and the paralogous replacement alleles, *Hoxa3*^{D3} and *Hoxd3*^{A3}. The null allele of *Hoxa3* contains a translation stop codon in the first exon and a frameshift mutation in the second exon. The *Hoxd3* null allele contains a deletion/insertion encompassing the homeodomain-coding region of the second exon. The *Hoxa3*^{D3} allele was generated by replacing the protein-coding domains of both *Hoxa3* exons with the corresponding sequences from the *Hoxd3* gene. All sequences flanking the protein-coding domains, 5' of the ATG, the entire intron and 3' of the TGA termination codon, are from the *Hoxa3* locus. Similarly, the *Hoxd3*^{A3} locus, in which the HoxA3 protein is synthesized from the *Hoxd3* locus, retains all the surrounding *Hoxd3* nucleotides. All alleles were introduced into their appropriate genomic loci by homologous recombination in murine ES cells²⁵. It should also be noted that the selectable markers (the *neo*^r genes), which are required for isolation of the targeted mutations, have all been replaced with a single, 32-base-pair (bp) *loxP* element²⁶, thus eliminating potential effects of marker gene expression on neighbouring genes.

Mice homozygous for null alleles at *Hoxa3* are characterized by perinatal lethality, absence of the thymus, and malformation of the hyoid bone^{17,18,20,21}. The glandular and skeletal abnormalities are exacerbated by mutations in the *Hoxd3* locus^{5,17,21}. To confirm that our null allele for *Hoxa3*, without the *neo*^r gene, conferred the originally described mutant phenotype, we crossed animals heterozygous for wild-type and null alleles (*Hoxa3*^{+/null} × *Hoxa3*^{+/null}). As shown in Table 1, no adult progeny of the *Hoxa3*^{null/null} genotype were recovered. As a test of whether the HoxD3 protein is sufficient to complement this *Hoxa3* deficiency, animals heterozygous for the wild-type and the *Hoxd3* transplacement alleles (*Hoxa3*^{D3/+}) were crossed with animals heterozygous for the wild-type and null alleles (*Hoxa3*^{+/null}). Adult progeny recovered from this cross represented all four possible genotypes at roughly normal mendelian frequencies (Table 1). The viability of the compound heterozygotes, *Hoxa3*^{D3/null}, shows that the HoxD3 protein, when expressed at the *Hoxa3* locus, complements the absence of HoxA3 protein.

To determine the extent of this complementation, embryos representing all *Hoxa3* allelic combinations were examined histologically. At embryonic day 17.5 (E17.5), the most striking abnormality of homozygous null (*Hoxa3*^{null/null}) embryos is the complete

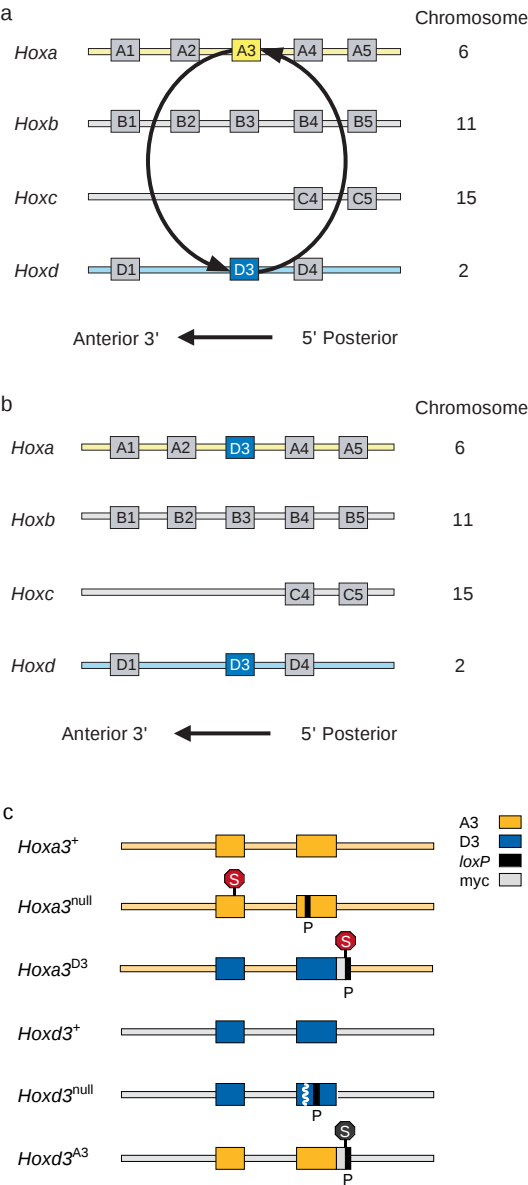


Figure 1 Representation of the wild-type (a) and one of the mutant Hox gene clusters (b). The individual alleles used are shown in (c). a, The 3' end of the wild-type Hox cluster, showing the relationship of paralogous genes. The black arrows indicate the paralogous protein-coding regions that were transplaced. b, The 3' end of the Hox cluster in animals that express the HoxD3 protein from the *Hoxa3* locus, referred to as the *Hoxa3*^{D3} allele. c, The six alleles used in this study. From top to bottom the alleles are wild-type *Hoxa3*, *Hoxa3*^{null}, *Hoxa3*^{D3}, wild-type *Hoxd3*, *Hoxd3*^{null} and *Hoxd3*^{A3}. Solid boxes represent protein-coding sequences, with those of HoxA3 and HoxD3 shown in dark yellow and dark blue, respectively. The *Hoxa* flanking non-coding sequences are in pale yellow, and *Hoxd* cis-acting regulatory sequences are in light blue. The homeodomain deletion in the *Hoxd3* null allele is white. Stop codons (S), *loxP* sites and the myc epitopes are indicated.

Table 1 *Hoxa3*^{D3} rescues the lethality of *Hoxa3*^{null/null}

Parental genotypes	<i>Hoxa3</i> ^{+/null} × <i>Hoxa3</i> ^{+/null}			
Possible genotypes of progeny	+/+	+ / null	null / null	
Number of progeny observed	13	28	0	
Parental genotypes	<i>Hoxa3</i> ^{+/null} × <i>Hoxa3</i> ^{D3/+}			
Possible genotypes of progeny	+/+	+ / null	D3 / +	D3 / null
Number of progeny observed	12	13	18	10

Table 2 Fraction of mice exhibiting normal glandular and skeletal structures

Genotype at the <i>Hoxa3</i> locus	+/+	null/null	D3/null	D3/D3	
Thymus	4/4	0/5	5/5	3/3	
Hyoid	18/18	0/7	11/11	6/6	
Genotype at the <i>Hoxd3</i> locus	+/+	+/null	null/null	A3/A3	A3/null
Anterior arch of the atlas	8/8	10/10	0/7	10/10	8/9
Dens	8/8	9/10	1/7	10/10(9/10)*	9/9 (6/9)*

* Indicates number of mice in which complementation was complete. Incomplete complementation of the *Hoxd3* null mutation by a single copy of *Hoxd3*^{A3} may result from a number of independent factors. It could represent the 15% haplo-insufficiency normally seen in *Hoxd3* heterozygotes¹⁹ (10% in this study), it may reflect a depression in protein activity due to the myc-epitope tag at the carboxy terminus of the *Hoxd3*^{A3} protein, and/or it may reflect slight differences in the biological activities of HoxA3 and HoxD3 in bone morphogenesis.

absence of the thymus (Fig. 2, Table 2). However, replacement of one or both copies of HoxA3 protein with the HoxD3 protein restores this organ. The effect of the various *Hoxa3* mutations on the throat cartilage was also assessed. Alterations of the hyoid, characteristic of embryos homozygous for the null allele of *Hoxa3*, are reversed by expression of the HoxD3 protein at the *Hoxa3* locus, as shown in Fig. 2 and summarized in Table 2. Moreover, adult animals lacking the HoxA3 protein but carrying one or two copies of the *Hoxa3*^{D3} allele appear anatomically completely normal (data not shown).

A conclusion from this data is that the HoxD3 protein is functionally equivalent to the HoxA3 protein if it is expressed in the context of the *Hoxa3* gene. A corollary to this conclusion is that the HoxA3 protein, if expressed at the *Hoxd3* locus will be unable to complement null mutations at *Hoxa3*. To test this, we crossed mice heterozygous for the *Hoxa3* mutation, *Hoxa3*^{+/-}, with mice heterozygous for the same mutation but that also carried the HoxA3 protein-coding domains inserted at the *Hoxd3* locus (*Hoxa3*^{+/-}; *Hoxd3*^{A3/+}). No adult progeny homozygous for the *Hoxa3* null allele were ever recovered from this cross. However, five dead newborn animals of the genotype *Hoxa3*^{null/null}; *Hoxd3*^{A3/+} were analysed for the status of the throat bones and cartilage. As shown in Fig. 2, these structures were indistinguishable from those found in the *Hoxa3*^{null/null} embryos, showing that when the HoxA3 protein is expressed from the *Hoxd3* locus, it is incapable of rescuing the *Hoxa3* mutant phenotype. Embryos of the same genotype,

Hoxa3^{null/null}; *Hoxd3*^{A3/+}, were also examined histologically, and no thymus was detected (Fig. 2).

Hoxd3 is required for the proper development of the cervical vertebrae¹⁹. Mice homozygous for a *Hoxd3* null mutation lack the anterior arch of the atlas and the dens of the axis. To assess the ability of the HoxA3 protein to function during vertebral morphogenesis, we replaced the protein-coding sequences of the *Hoxd3* gene with *Hoxa3* protein-coding sequences, leaving all adjacent, non-coding sequences intact. We were able to generate viable, fertile adult animals in which the three *Hoxd3* alleles, *Hoxd3*⁺, *Hoxd3*^{null} and *Hoxd3*^{A3}, were represented in all six possible combinations. Skeletal analyses were carried out on adult animals of each genotype with emphasis on the structure of the atlas and axis (Fig. 3, Table 2). Mice homozygous for the null allele lack the anterior arch of the atlas as well as the dens. Both of these structures were restored by the presence of two copies of the *Hoxd3*^{A3} allele. A single copy of the *Hoxd3*^{A3} allele also complements the mutant phenotype, but with incomplete penetrance. In three of nine animals of the *Hoxd3*^{A3/null} genotype, the dens was fused (either completely or partially) to the anterior arch of the adjacent atlas; in one of these same individuals, the arch itself was incompletely formed. Neither the atlas nor axis phenotype was suppressed by the expression of the HoxD3 protein at the *Hoxa3* locus (data not shown), showing again the critical role of *cis*-acting sequences in determining the functional specificity of the gene products.

The hybrid alleles generated in this study are characterized by the precise engineering that distinguishes between coding and non-coding sequences, and by the targeting of single copies of the coding moieties to the chromosomal loci of their paralogues. Promoters, enhancers, intron-encoded splicing signals and 3' mRNA processing signals are thus the ones that are normal for the host locus. We propose that this provides the most precise means of retaining the normal temporal, spatial and quantitative parameters of gene expression.

The successful bi-directional complementation of both the *Hoxa3* and *Hoxd3* genes has proved informative on several counts. First, it shows that these proteins, which share less than 50% identity in amino-acid sequence, are capable of carrying out equivalent biological functions in the processes recognized to require *Hox3* gene activity. Second, it provides direct evidence that the different roles played by these genes during embryogenesis are mainly the result of

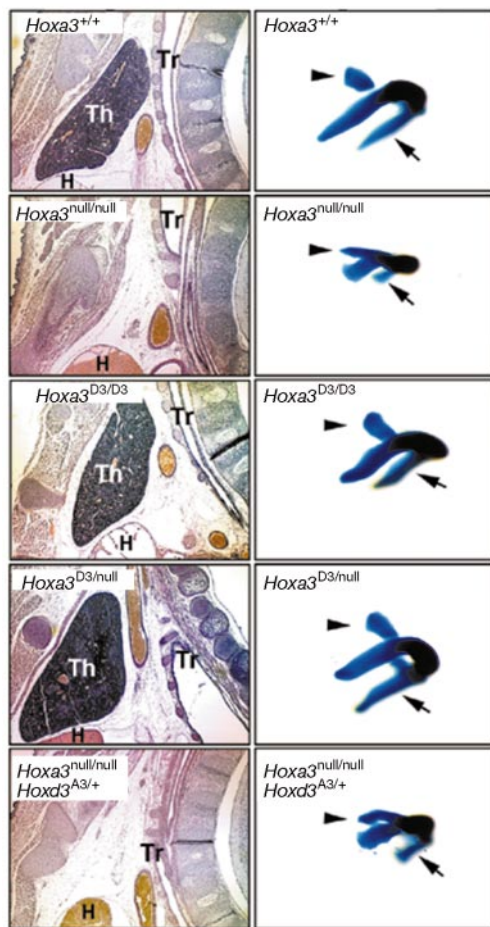


Figure 2 Thymus and hyoid phenotypes of *Hoxa3* null mutants and complementation by the *Hoxa3*^{D3} allele. Left, parasagittal sections from the throat regions of E17.5 embryos. Right, dissected hyoid bones from either E18.5 embryos or newborn animals. Arrows and arrowheads indicate the lesser and greater horns of the hyoid bone, respectively. Th, thymus; Tr, trachea; H, heart.

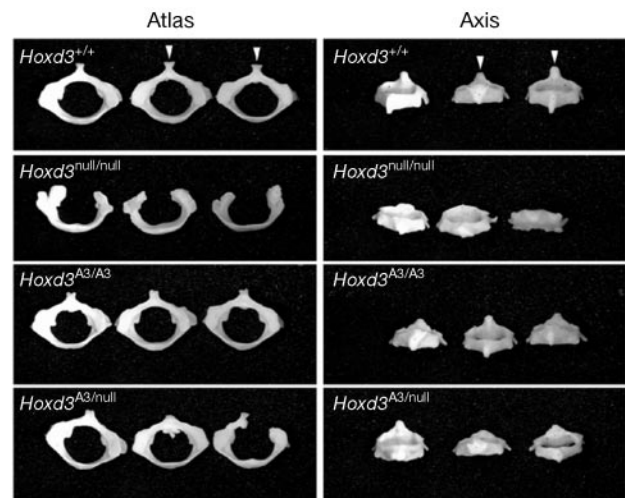


Figure 3 Atlas and axis phenotypes of *Hoxd3* null mutants and complementation by the *Hoxd3*^{A3} allele. The atlas and axis from three animals of each genotype are shown. The atlas and axis occupying the same relative positions in each panel are from the same animal. Arrowheads denote the anterior arch of the atlas (left panel) and the dens of the axis (right panel).

cis-acting sequences that modulate expression of the individual loci. Whether equivalent scenarios hold for other paralogous Hox gene families remains to be tested. We want to stress that the non-equivalence of *Hoxa3* and *Hoxd3* in their normal, wild-type, chromosomal environments may not be due to qualitative differences in the spectrum of cell-specific expression. The expression patterns of *Hoxa3*, *Hoxb3* and *Hoxd3* are very similar, if not completely overlapping. Rather, we suggest that the important parameter controlling, for example, the differential function of *Hoxa3* and *Hoxd3* in mesenchymal neural crest and somitic mesoderm, respectively, may be that each gene is expressed in particular cell types at sufficient levels to activate its respective developmental programme; *Hoxa3* being expressed at a higher concentration in one context and *Hoxd3* in another. In this model, qualitative differences in the behaviour of genes are the consequence of quantitative differences in their expression levels. Further, the cross-exacerbations of the mutant phenotypes in the double mutants argue for co-expression of these paralogues in all the tissues of the neck region and that they function coordinately. At the molecular level, these observations suggest that these Hox genes mediate their functions by collectively controlling common target genes through conserved target *cis* elements. The outputs from the target genes would thus reflect signals generated from the combined binding activities of all of the paralogous Hox3 products. The sensitivity of these developmental programmes to the overall combined concentration of paralogous gene products is demonstrated by the observation that, although mice heterozygous for individual *Hox3* paralogous genes are outwardly normal, most mice heterozygous at all three *Hox3* loci die perinatally¹⁷.

It is remarkable that in the estimated 530 million years since quadruplication of the Hox complex in early vertebrates, the near perfect functional equivalence described here has been maintained for the HoxA3 and HoxD3 proteins. We suggest that this conservation of functional equivalence may not be an accident of evolution, but rather an indication that equivalence of function is at the very core of normal Hox gene function during development, perhaps facilitating a necessary quantitative interaction among these proteins. □

Methods

Allele generation

The null allele of *Hoxa3*, *Hoxa3*^{null}, has been described²⁶. It contains an 8-bp insertion at the unique *Eco47III* site in exon 1 and a *loxP*-containing insertion at the *BglIII* site in the homeodomain of exon 2. The null allele of *Hoxd3*, *Hoxd3*^{null}, contains a *loxP* site instead of the 249 nucleotides between the two *BglIII* sites in exon 2. This mutant allele was created by the self-excising *neo*^c cassette, ACN, also used to generate *Hoxa3*^{null}. The exchange of coding sequences required to generate *Hoxa3*^{D3} and *Hoxd3*^{A3} first necessitated the sequencing of all junctions between coding and non-coding regions of both genes. The sequences are as follows:

Hoxa3 exon 1, 5'-aaacatcgcgATGCAAAAAG.....TCCAGCTCAGtgaatgaat-3';

Hoxa3 exon 2, 5'-gtcctggcagGGGAGAGCTG.....CACCCACCTGtgatgatggg-3';

Hoxd3 exon 1, 5'-tgagtgcaccATGCAGAAGG.....GCCACTTCAGtgattctct-3';

Hoxd3 exon 2, 5'-ccttgccagGAGAGAACTG.....GACACATCTGtagctgtggc-3';

where lower and upper case letters indicate non-coding and coding sequences, respectively, and start- and stop-translation codons are indicated by bold type and underline, respectively.

Restriction endonuclease cleavage sites were identified in all regions 5' and 3' to each coding region and used to remove the coding sequences and linked nucleotides from cloned copies of the two genes. Combinations of synthetic oligonucleotides, restriction- and endonuclease-generated fragments and PCR-generated fragments were used to reconstruct the transplanted coding sequences and any missing host, flanking nucleotides. These were then re-inserted into the appropriate sites. Exons and flanking sequences were sequenced before construction of the final targeting vectors. Both the *Hoxa3*^{D3} and *Hoxd3*^{A3} coding regions were fused at the 3' end to an 11-amino-acid c-myc epitope, and a *loxP*-flanked *neo*^c gene was inserted immediately 3' of the termination codon in exon 2 of each gene. The alleles were introduced into mouse ES cell line RI²⁷ and subjected to positive-negative selection²⁸ before identification of recombinant cell lines. Two cell lines representing each allele were used to generate mice by blastocyst injection. The *neo*^c marker

gene was removed by mating the progeny of the chimaeric animals with a *Cre*-expressing 'deleter mouse'²⁹ before colony expansion and phenotypic analysis.

Phenotypic analysis of embryos

The characterization of the pharyngeal organs in E17.5 embryos was carried out after serial sectioning as previously described³⁰. Embryos from timed pregnancies were fixed in Bouin's reagent for 4 days, rinsed in a sucrose/phosphate solution and hemisected with a razor blade. The samples were then embedded in paraffin, sectioned sagittally (10 µm) and stained on glass slides with haematoxylin and eosin. E18.5 day embryos used for skeletal analysis were fixed in ethanol, stained with alizarin red and alcian blue, and cleared in glycerol as described⁶.

Preparation of adult skeletons

Six- to ten-week-old adult animals were killed by CO₂ asphyxiation and the head and cervical region removed. After removal of the skin, the samples were boiled for 5 min in water and incubated overnight in a solution of 0.5% trypsin and 30% borax. The sample was boiled again in water for 10 min, and the cleaned bones stored in 95% ethanol.

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The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences

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Campylobacter jejuni, from the delta-epsilon group of proteobacteria, is a microaerophilic, Gram-negative, flagellate, spiral bacterium—properties it shares with the related gastric pathogen *Helicobacter pylori*. It is the leading cause of bacterial food-borne diarrhoeal disease throughout the world¹. In addition, infection with *C. jejuni* is the most frequent antecedent to a form of neuromuscular paralysis known as Guillain-Barré syndrome². Here we report the genome sequence of *C. jejuni* NCTC11168. *C. jejuni* has a circular chromosome of 1,641,481 base pairs (30.6% G+C) which is predicted to encode 1,654 proteins and 54 stable RNA species. The genome is unusual in that there are virtually no insertion sequences or phage-associated sequences and very few repeat sequences. One of the most striking findings in the genome was the presence of hypervariable sequences. These short homopolymeric runs of nucleotides were commonly found in genes encoding the biosynthesis or modification of surface structures, or in closely linked genes of unknown function. The apparently high rate of variation of these homopolymeric tracts may be important in the survival strategy of *C. jejuni*.

Human infection is usually acquired by the consumption of contaminated food (especially poultry) or water¹. Motile campylobacters colonize the intestines of a wide range of animals, but in immunologically naive humans infection frequently results in an inflammatory enterocolitis. The number of cases of *Campylobacter* infection reported in England and Wales in 1998 increased by 17% from the previous year, with the number of reported cases now more than double that due to *Salmonella*³. Despite its importance, effective control of *Campylobacter* in the food chain and the design

of disease prevention strategies are hindered by a poor understanding of the genetics, physiology and virulence of this organism.

The genome of *C. jejuni* NCTC11168 is 1,641,481 base pairs (bp) in length. Of the 1,654 predicted coding sequences (CDS), at least 20 probably represent pseudogenes; the average gene length is 948 bp, and 94.3% of the genome codes for proteins, making it the densest bacterial genome sequenced to date. The bias towards G on the leading strand of the chromosome⁴ indicates that the origin of replication is near to the start of the *dnaA* gene. Strand bias is also evident in the CDSs; overall, 61.1% are transcribed in the same direction as replication (Fig. 1). We discovered two large regions of lower G+C content that encompass CDSs Cj1135–Cj1148 (25.4%) and Cj1421–Cj1442 (26.5%); these correspond to genes within the lipooligosaccharide (LOS) and extracellular polysaccharide (EP) biosynthesis clusters, respectively. Functional information (matches to genes of known function, or informative hydrophobicity profiles) could be deduced for 77.8% of the 1,654 CDSs, whereas 13.5% matched genes of unknown function in the database and 8.7% had no database match, or other functional information. The unusually low number of unknowns reflects the preponderance of predicted membrane, periplasmic and lipoproteins; these make up 10.3%, 7.8% and 2.3% of the CDSs, respectively, and many of these have no database matches.

One surprising feature of the *C. jejuni* genome is the almost complete lack of repetitive DNA sequences. In fact, there are only four repeated sequences within the entire genome; three copies of the ribosomal RNA operon (6 kilobases (kb)) and three duplicated or triplicated CDSs. Apart from Cj0752, which is similar to part of IS605 *tnpB* from *H. pylori*, there is no evidence of any functional inserted sequence (IS) elements, transposons, retrons or prophages

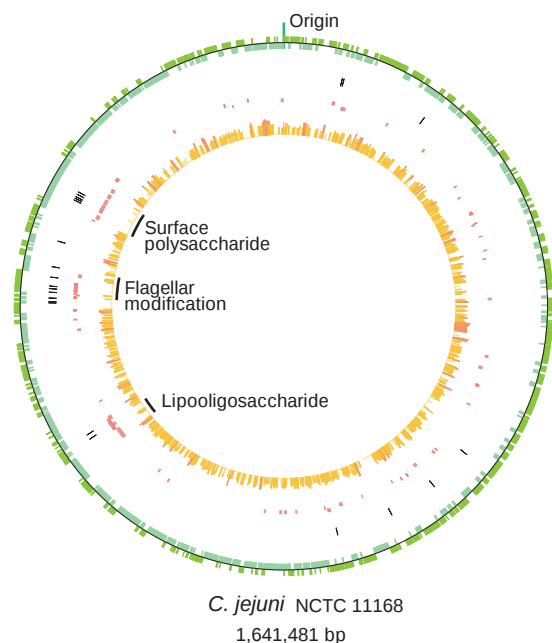


Figure 1 Circular representation of the *C. jejuni* genome. From the outside to the inside, the first circle shows coding sequences transcribed in the clockwise direction in dark green; the second shows coding sequences in the anticlockwise direction in pale green. The putative origin of replication is marked. The third shows the positions of hypervariable sequences in black, and the fourth and fifth show genes involved in the production of surface structures: clockwise in dark red and anticlockwise in pale red. The innermost histogram shows the similarity of each gene to its *H. pylori* orthologue, where present; the height of the bar, and the intensity of the colour, are proportional to the degree of similarity. The clusters of genes responsible for LOS biosynthesis, EP biosynthesis and flagellar modification are marked.

in the genome. Another intriguing feature of the genome is that, apart from salient exceptions such as the two ribosomal protein operons and gene clusters involved in LOS biosynthesis, EP biosynthesis and flagellar modification, there appears to be little organization of genes into operons or clusters. Although genes do fall into long, apparently linked sets, generally the genes within these sets appear to be functionally unrelated. The distribution of the genes involved in amino-acid biosynthesis, for example, reflects this organization: some of the *his*, *leu* and *trp* genes are apparently organized into operons, but the *aro*, *asp*, *dap*, *gln*, *gly*, *ilv*, *met*, *phe*, *pro*, *ser*, *thr* and *tyr* genes are scattered randomly throughout the genome.

The shotgun assembly revealed regions in which the sequences of otherwise identical clones varied at a single point. These were mainly, but not exclusively, length variations in polyG:C tracts (Table 1). The degree of variation differs between sites, and there are homopolymeric tracts that do not display variability within the observed shotgun sequences (Table 2). Variation in the length of polyG:C tracts is frequently associated with contingency genes in other pathogenic bacteria, and may be produced by slipped-strand mispairing during replication⁵; it can affect translation and has been shown to be responsible for phase variation of surface properties or antigenicity⁶. The appearance of variants due to slipped-strand mispairing occurs in *Neisseria meningitidis* with a frequency of 10^{-3} per cell per generation⁷; these variants are therefore likely to be rare in the sampled clones of a 10-fold shotgun library derived from a clonal population. The *C. jejuni* sequence, by contrast, shows some regions where three or more variants are present in almost equal proportions, in addition to many regions where two variants are present. The absolute rate of variation is difficult to estimate from these data, although the number of changes seen suggests that the frequency is much higher than in other organisms.

The variation in homopolymeric tracts was not an artefact of the sequencing process, as fourfold resequencing of several variants did not show any difference from the original sequence. A shotgun sequence of an appropriate lambda clone demonstrated that the variation did not occur during subcloning in *Escherichia coli*. Support for the existence of rapid phase variation in *C. jejuni* is also provided by *H. pylori* J99, for which similar variation was seen⁸. This rapid sequence variation suggests that *C. jejuni* may be lacking in DNA repair functions, indeed, many DNA repair genes studied in *E. coli* cannot be found in *C. jejuni*, including the direct repair genes *ada* and *phr*; the glycosylases *tag*, *alkA*, *mutM* and *nfo*; the mismatch repair genes *vsr*, *mutH*, *mutL* and *sbcB*; and the SOS response genes

lexA, *umuC* and *umuD*. Significantly, transcription-coupled repair may influence the rate of phase variation in *N. meningitidis*⁷. The high levels of variation seen in the shotgun sequences mean that it is not possible to produce a single definitive sequence for the *C. jejuni* genome. As such, it possesses some of the properties of a quasi-species; a phenomenon that is well described in RNA viruses⁹.

Most of the hypervariable sequences cluster on the genome and are coincident with the clusters of genes responsible for LOS biosynthesis, EP biosynthesis and flagellar modification (Fig. 1). Some of the variable genes can be ascribed putative functions, such as glycosyl-transferases; several others belong to two families (designated as 617 and 1318; Fig. 2, Table 3). The 617 family of genes have no homologues outside *C. jejuni*, while the 1318 family has two homologues in *H. pylori* (HP0114 and HP0465). Sixteen additional members of the 1318 family occur in various bacterial and archaeal species, none with a well-defined function; however, family members within the enterobacteriaceae are found within lipopolysaccharide gene clusters, supporting our hypothesis that these proteins are involved in the synthesis of surface structures. The rapid variation in surface properties implied by these results may have more relevance to the colonization of a dynamic intestinal environment than to immune avoidance.

C. jejuni has been reported to produce a variety of toxins whose activity and/or role in pathogenesis remain controversial¹⁰. The genome of NCTC11168 does not contain a cholera-like toxin gene, although genes encoding the cytolethal distending toxin (*cdtA-C*) are present. A member of the family of contact-dependent haemolysins found in pathogenic *Serpulina* and *Mycobacterium* species¹¹ (Cj0588), a putative integral membrane protein with a haemolysin domain (Cj0183) and a phospholipase (*pldA*) were also identified.

In contrast to most lipid A and some inner core biosynthesis genes, many LOS biosynthesis proteins are encoded by a large gene cluster (Cj1119–Cj1152) which has a role in core biosynthesis and also protein glycosylation^{12,13}. There is increasing evidence that *C. jejuni* synthesizes an EP that is not attached to lipid A¹³, and thus the presence of genes similar to those involved in bacterial capsule biogenesis is significant. Two groups containing *kps* orthologues (involved in transport; A.V.K., manuscript submitted) were found and the region between the groups contains many different polysaccharide biosynthetic genes, some required for the biosynthesis of bacterial capsules. As might be expected with the presence of *kps* genes, no orthologues to *wzx*, *wzy* and *wzz* genes, which are essential for heteropolymeric O-chain biosynthesis, are present. Unusually, *C. jejuni* has three sets of *neu* genes involved in sialic

Table 1 Hypervariable sequences found in the *C. jejuni* genomic shotgun

Polymorphism	No. of clones with each variant	Gene(s) affected	Putative function	Effect
G(8–10)	10=14/20, 9=3/20, 8=3/20	Cj0031/Cj0032	Probable restriction/ modification enzyme	Fusion/separation
C(9–11)	11=6/21, 10=13/21, 9=2/21	Cj0045c	Haemerythrin-like putative iron-binding protein	Extension and overlap
G(9–13)	13=1/13, 12=3/13, 11=4/13, 10=4/13, 9=1/13	Cj0046	Pseudogene (transport protein)	None
G(9,11)	9=6/7, 11=1/7	Cj0170/ Cj0171	Unknown, similar to Cj1325/Cj1326	Fusion/separation
T(4–5)	5=7/9, 6=2/9	Cj0628/Cj0629	Lipoprotein	Fusion/separation
G(9–10)	9=7/9, 10=2/9	Cj0685c	Possible sugar transferase	Truncation/extension
G(10–11)	10=5/9, 11=4/9	non coding	Upstream of rRNA	None apparent
C(8–9)	8=12/15, 9=3/15	Cj1139c	Galactosyltransferase	Truncation/extension
C(8–9)	8=2/3, 9=1/3	Cj1144c/ Cj1145c	Unknown	Fusion/separation
C(9–10)	9=7/8, 10=1/8	Cj1305c	Unknown, 617 family	Truncation/extension
C(8–9)	8=4/10, 9=6/10	Cj1306c	Unknown, 617 family	Truncation/extension
C(9–10)	9=4/5, 10=1/5	Cj1310c	Unknown, 617 family	Truncation/extension
G(10–11)	10=6/7, 11=1/7	Cj1318	Unknown, 1318 family	Truncation/extension
G(10–11)	10=5/9, 11=4/9	upstream of Cj1321	Transferase	None apparent (promoter?)
G(9–10)	9=2/14, 10=12/14	Cj1325/Cj1326	Unknown	Fusion/separation
G(9–10)	9=7/8, 10=1/8	Cj1335/Cj1336	Unknown, 1318 family	Fusion/separation
C(9–10)	9=9/11, 10=2/11	Cj1342c	Unknown, 617 family	Truncation/extension
C(1–2)	1=2/10, 2=8/10	Cj1367	Possible nucleotidyltransferase	Truncation/extension
C(9–10)	9=5/8, 10=3/8	Cj1420c	Possible methyltransferase	Truncation/extension
C(8–10)	8=1/9, 9=6/9, 10=2/9	Cj1421c	Unknown, similar to putative sugar transferases	Truncation/extension
C(9–10)	9=9/10, 10=1/10	Cj1422c	Unknown, similar to putative sugar transferases	Truncation/extension
C(10–11)	10=10/11, 11=1/11	Cj1426c	Unknown	Truncation/extension
C(9–10)	9=1/10, 10=9/10	Cj1429c	Unknown	Truncation/extension

acid biosynthesis. Sialic acid is an uncommon constituent of bacterial surface structures and, through molecular mimicry, may be important for evasion of host immunity and in post-infection autoimmune diseases such as Guillain–Barré syndrome. A complete cluster (*neuB1*, *C1*, *A1*) has a role in LOS sialylation (D. Linton *et al.*, personal communication). Another set (*neuB2* and *C2*) is in close proximity to *ptmAB* and may be involved with these genes in post-translational modification of flagellin¹⁴.

There are no obvious orthologues of the extensive Hop porin family of *H. pylori*¹⁵ and, contrary to a recent report¹⁶, no type III secretion systems were identified other than the flagellin export apparatus. In the absence of such a system, it is possible that the CiaB protein¹⁶ is secreted by the flagellin export apparatus¹⁷. In contrast to *Campylobacter fetus* and *Campylobacter rectus*, genes encoding S-layer proteins were apparently absent in *C. jejuni*. Under certain conditions, *C. jejuni* produce 4–7 nm wide filaments resembling pili¹⁸; although structural pilin orthologues appear to be absent, there are several type 4 pilus related genes such as a prepilin peptidase (Cj0825) and several putative type II export genes (Cj1470c–Cj1474c). Flagella are one of the best-defined virulence factors, as *flaA* and *flaB* mutants are markedly reduced in virulence¹⁹. Whether this is due to a direct involvement of the flagellin, or perhaps an inability to secrete proteins through the flagella export apparatus, is unknown¹⁷. Two further structural flagellin paralogs were identified (*flaC* Cj0720c and *flaD* Cj0887c), and orthologues of the majority of flagellar-associated genes of clearly understood function in bacteria such as *S. typhimurium* are represented; however, several genes involved in regulation are absent, including *flhCD*, *flgM*, *fliT* and *fliK*. These differences from the enterobacterial paradigm mirror those found in *H. pylori*. However, the gene encoding the *H. pylori* flagellar sheath protein *hpaA* (ref. 20) is absent in *C. jejuni*, as is a flagellar sheath.

Chemotaxis is important for intestinal colonization by *C. jejuni*. Like *H. pylori*, *C. jejuni* produces three proteins containing the response regulatory domain of CheY. One is CheY (Cj1118)²¹, and the others are found fused to a histidine protein kinase domain (CheA) or a CheW-like domain (CheV). In contrast to *H. pylori*, only one CheV orthologue is present. The regulation of CheY

activity in *C. jejuni* is different from that of *H. pylori* as, unlike *H. pylori*, orthologues of both CheB and CheR are present. No orthologues of CheZ or other chemotaxis genes were found. Ten genes contain methyl-accepting chemotaxis protein domains; these are candidate chemoreceptor genes, some of which may transduce signals to non-taxis-associated pathways; only three (Cj1506, Cj0448, Cj0019) have orthologues in *H. pylori*.

The genome encodes five major iron-acquisition systems, which are mostly organized in operons under the control of the Fur protein²². Two of these iron-acquisition systems, the enterochelin uptake operon *ceuBCDE* and the siderophore receptor orthologue *cfrA*, have been described previously. In addition, there is a predicted haemin uptake operon *chu* (Cj1614–1617)²², a periplasmic binding protein dependent system (Cj0173c–0175c) and a siderophore receptor (Cj0178) with accessory genes. Three copies of the accessory *tonB*, *exbB* and *exbD* genes, which form the energy transduction machinery for transport of iron compounds over the outer membrane, were found. One of the *exbB-exbD-tonB* triplets follows the Cj0178 siderophore receptor, whereas another *tonB* gene is divergently orientated to *cfrA*. *C. jejuni* might therefore use separate energy transduction mechanisms for transport of the different iron substrates, as has been shown for *Vibrio cholerae*.

C. jejuni appears to have a broader repertoire of regulatory systems than *H. pylori*, which has a similar sized genome. Given that *C. jejuni* is found in a more diverse range of ecological niches than *H. pylori*, this might be expected. The apparent lack of operon organization raises fundamental issues concerning transcriptional regulation in *Campylobacter*. Although it is possible that each gene is independently transcribed, strand-specific gene grouping suggests co-transcription. Like *H. pylori*, the *Campylobacter* genome contains only three predicted sigma factors (*rpoD*, *rpoN* and *fliA*). The largest proportion of regulatory genes consists of members of the two-component regulator family. As in *Bacillus subtilis*, *C. jejuni* has an additional member of the Fur²² family, PerR, which regulates the peroxide stress regulon²³. Unlike *H. pylori*, *C. jejuni* contains a possible *crp/fnr* family member (Cj0466) with both helix–turn–helix and cNMP-binding motifs.

As might be expected from the inability of *C. jejuni* to use carbohydrates as carbon or energy sources, very few genes for degradation of carbohydrates or amino acids were detected. The glycolytic pathway is also apparently incomplete; orthologues of the glucokinase and 6-phosphofructokinase genes were not found, although these functions may well be supplied by non-orthologous genes. Despite this, *C. jejuni* does appear to have all the genes necessary for gluconeogenesis and, unlike *H. pylori*, *C. jejuni*

Table 2 Potentially variable homopolymeric tracts

Sequence	Gene(s) affected	Putative function	Potential effect
G(8)	Cj0275 (<i>clpX</i>)	Clp protease ATP-binding subunit	Truncation
G(12)	Cj0565	Non-coding, upstream of pseudogene	None
G(9)	Cj0617/Cj0618	Unknown, 617 family	Fusion/separation
G(10)	Cj0628/Cj0629	Lipoprotein (adjacent to variable T(4–5) sequence)	Fusion/separation
G(9)	Cj0676 (<i>kdpA</i>)	Pseudogene (potassium-transporting ATPase A chain)	None
G(9)	Cj1295	Unknown	Truncation
G(9)	Cj1296/Cj1297	Weak similarity to aminoglycoside N3'-acetyltransferases, similar to Cj1298	Fusion/separation
C(9)	Cj1437c	Aminotransferase	Truncation
T(7)	Cj1677/Cj1678	Unknown, similar to Cj0628/Cj0629	Fusion/separation

Table 3 The largest paralogous gene families in *C. jejuni*

No. of genes	Function
28	ABC transporter ATP-binding proteins
17	Sugar transferases
14	Binding-protein-dependent permeases
12	Two-component regulators
10	MCP-domain-containing proteins
8	Sugar epimerases/dehydratases
7	1318 family
7	'Hexapeptide'-type transferases
7	Two-component sensors (histidine kinases)

MCP, methyl-accepting chemotaxis protein.

617 family

```

161
Cj617 EYQNFQIMQAMDILNAIFYIKENSPFKLMRCG-----210
Cj618 EYQNFQIMQAMDILNAIFYIKENSPFKLMRCG-----
Cj1305 DYQNYGIMAAIDHINALKDLVKRFP...KFAADLPKIYGGGSGYGYLALLI
Cj1306 EYQNFQIMAAIDHINALKDLVKRFP...KLADLPKIYGGGSGYGYLALLI
Cj1310 DYQNYGIMAAIDHINALKDLVKRFP...KFAADLPKIYGGGSGYGYLALLI
Cj1342 EYQNFQIMQAQDLNVALYLLKKHAPFDTHGGGPIIMIGGSHGGYLAHLA
Cons. EYQNFQIM-A-D-INAL--L-K-P-----LP-I--GGSYGYL--L-

```

1318 family

```

56
Cj1318 SIKNNGGGYNENLLYQDPIKELQTMNTYNDKYLIPVLYFYGFNGNIGL105
Cj1333 SDNTFLYE.....NVIDELNSMLTYNDKYLIPVLYFYGFNGNIGL
Cj1334 QDENGINFKDDIFLYENPNKELLENLTFLKTEYNKYPPVLYFYGFNGMF
Cj1335 SIKNNGGG-----
Cj1336 SIKNNGGGYNENLLYQDPIKELQTMNTYNDKYLIPVLYFYGFNGNIGL
Cj1337 IIKKR...NLKKMYQDPIKEKLENYFKD.FTRYPPVLYFYGFNGNIGL
Cj1340 DENLNIFDKTHNVFMYENLEEEINFFYQSILEKTPRYPFICIFYGIGNALL
Cj1341 DENLNIFDKTHNVFMYENLEEEINFFYQSILEKTPRYPFICIFYGIGNALL
Cons. -----Y-----EL-----D----YP-L--YG-GN-IL

```

Figure 2 Multiple alignments of partial sequences of the 617 and 1318 gene families. Amino acids encoded by the hypervariable homopolymeric tracts are in boxes. Cj0617 and Cj0618, and Cj1335 and Cj1336 represent coding sequences frameshifted at the homopolymeric tract.

appears to encode an intact tricarboxylic acid cycle (like *H. pylori*, *C. jejuni* uses 2-oxoglutarate ferredoxin oxidoreductase (OorDABC)²⁴, rather than 2-oxoglutarate dehydrogenase to interconvert 2-oxoglutarate and succinyl CoA).

C. jejuni and *H. pylori* are closely related by 16S rRNA phylogeny, share many biological properties and were previously classified within the *Campylobacter* genus. Figure 1 (and Supplementary information) indicates which *C. jejuni* genes are present or absent in *H. pylori* 26695 (ref. 15). The three polysaccharide biosynthetic loci relating to surface structure stand out as being unique to *C. jejuni* and are correlated with a high density of polymorphic sequences. *C. jejuni* also contains a large number of biosynthetic genes not present in *H. pylori*, such as those directing the synthesis of purines, thiamine and many amino acids. Genes present in *H. pylori* but absent in *C. jejuni* include the urease operon, nickel transport system, vacuolating toxin and the Cag pathogenicity island¹⁵. These features are consistent with the unique niche of *H. pylori* in the stomach, and its pathophysiology and propensity for chronic infection.

Despite the close phylogenetic relationship of *C. jejuni* and *H. pylori*, strong similarities between them are mainly confined to housekeeping functions; only 55.4% of *C. jejuni* genes have orthologues in *H. pylori*. In most functions related to survival, transmission and pathogenesis, the organisms have remarkably little in common. This indicates that selective pressures have driven profound evolutionary changes to create two very different and specific pathogens appropriate to their niches, from a relatively close common ancestor. Overall, 28.0% of genes show closest similarity to genes from *E. coli*, 27.0% to genes from *B. subtilis*, 4.6% to genes from *Archeoglobus fulgidus* and 2.1% to genes from *Saccharomyces cerevisiae*. Several genes were found which have homologues only in the eukaryotic domain; these include the dUTPase (*dut*) gene (Cj1451), which is similar to those of *Leishmania major* and *Trypanosoma cruzi* only; there is no orthologue of the *E. coli* *dut* gene. Taken together, these statistics suggest that the evolution of the *C. jejuni* genome does not neatly mirror that of its small subunit ribosomal RNA, and that the placement of *C. jejuni* within the Gram-negative proteobacteria may rely on a simplified view of the evolutionary origin of its genome.

This genomic sequence provides the resources for a complete and detailed analysis of the pathogenic potential of this enigmatic pathogen. New insights into the biology of *C. jejuni* include the identification of hypervariable sequences, lack of classical operon structure and repetitive DNA, and an unexpected capacity for polysaccharide production. □

Methods

A single colony of *C. jejuni* (NCTC11168, human origin, serotype O2, minimally passaged) was spread on one petri dish of CCDH agar (*Campylobacter* selective agar, Oxoid), and re-streaked on 10 CCDH agar plates. Cells were harvested and total DNA (10 mg) was isolated using proteinase K treatment followed by a phenol extraction procedure. The DNA was fragmented by sonication, size-fractionated on an agarose gel, and seven libraries were generated in pUC18 using size fractions ranging from 1.0 kb to 2.2 kb. Roughly 19,400 pUC clones were sequenced from both ends, using Dye-terminator chemistry on ABI 373 and 377 sequencing machines. The final assembly was generated from 33,900 reads, giving an ~10-fold coverage of the genome. Sequence assembly was accomplished using Phrap (P. Green, unpublished), and the sequencing was finished using GAP4²⁵. The assembly was verified by genomic PCR reactions across all repeats, in addition to 130 forward and reverse reads from a random library of ~10–16-kb fragments of genomic DNA cloned in lambdaFixII (Stratagene). In the final assembly, 0.13% of the genome was covered by a single clone only, and 0.11% was not sequenced on both strands, or with complementary sequencing chemistries.

The DNA was compared with sequences in the EMBL database using BLASTN and BLASTX²⁶. Transfer RNAs were predicted by tRNAscan-SE²⁷. Potential CDSs were predicted using ORPHEUS²⁸ and GLIMMER²⁹ (both trained on an initial open reading frame set generated by ORPHEUS), and also stop-to-stop prediction; the results were combined. The predicted protein sequences were searched against a non-redundant protein database using WUBLASTP and FASTA. The complete six-frame translation was used to search PROSITE, and the predicted proteins compared against the PFAM³⁰ database of protein domain hidden Markov models. The results of all these analyses were assembled together using the Artemis sequence viewer (K.M.R., unpublished) and used to

inform a manual annotation of the sequence and predicted proteins. Annotation was based, wherever possible, on characterized proteins or genes.

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Supplementary information is available on Nature's World-Wide Web site (<http://www.nature.com>) or as paper copy from the London editorial office of Nature.

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Shiga-like toxins are neutralized by tailored multivalent carbohydrate ligands

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The diseases caused by Shiga and cholera toxins account for the loss of millions of lives each year¹. Both belong to the clinically significant subset of bacterial AB₅ toxins consisting of an enzymatically active A subunit that gains entry to susceptible mammalian cells after oligosaccharide recognition by the B₅ homopentamer^{2,3}. Therapies might target the obligatory oligosaccharide–toxin recognition event⁴, but the low intrinsic affinity of carbohydrate–protein interactions hampers the development of low-molecular-weight inhibitors⁵. The toxins circumvent low affinity by binding simultaneously to five or more cell-surface carbohydrates⁶. Here we demonstrate the use of the crystal structure of the B₅ subunit of *Escherichia coli* O157:H7 Shiga-like toxin I (SLT-I) in complex with an analogue of its carbohydrate receptor⁶ to design an oligovalent, water-soluble carbohydrate ligand (named STARFISH), with subnanomolar inhibitory activity. The *in vitro* inhibitory activity is 1–10-million-fold higher than that of univalent ligands and is by far the highest molar activity of any inhibitor yet reported for Shiga-like toxins I and II. Crystallography of the STARFISH/Shiga-like toxin I complex explains this activity. Two trisaccharide receptors at the tips of each of five spacer arms simultaneously engage all five B subunits of two toxin molecules.

Shiga toxin, which is produced by *Shigella dysenteriae* type 1, and the homologous Shiga-like toxins (SLTs) of *E. coli*, also called Verotoxins (VTs), can cause serious clinical complications in

humans infected by these organisms^{7–9}. The functional toxin receptor on mammalian cells is the glycolipid Gb₃ (α-D-Gal(1 → 4)β-D-Gal(1 → 4)β-D-Glc(1 → O-ceramide)⁸ and the high incidence of complications such as acute kidney failure in children correlates with the expression of Gb₃ in the paediatric renal glomerulus¹⁰. Synthetic Gb₃ analogues covalently attached to insoluble silica particles competitively adsorb toxin from the gut and the adsorbent Synsorb P^k is currently undergoing clinical trials¹¹. Once the toxin has exited the gut and entered the circulation, however, there is no viable toxin therapy.

Shiga-like toxins from *E. coli* represent a family of structurally and functionally related toxins, which can be classified into two subgroups, SLT-I and SLT-II, on the basis of their relatedness to the prototypic Shiga toxin expressed by *S. dysenteriae*^{12,13}. Nevertheless, homology modelling suggests that structural changes in the binding sites are fairly conservative.

The crystal structure of the SLT-I B-subunit pentamer in complex with a Gb₃ trisaccharide analogue has been solved at 2.8 Å resolution⁶. Three saccharide-binding sites are found in each relative molecular mass 7,700 (*M_r* 7.7K) subunit. The subunits associate non-covalently to form a doughnut-shaped B-subunit pentamer with 15 saccharide-binding sites aligned on one face of the toxin. The A subunit is attached to the opposite surface of the

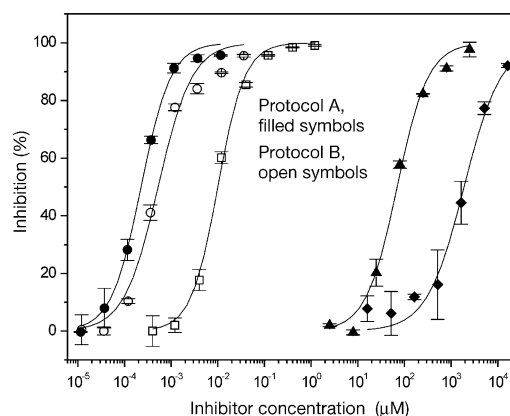


Figure 2 Inhibitor activity by enzyme linked immunosorbent assay (ELISA). Protocol A: diamonds, P^k trisaccharide 1 and SLT-I assay (IC₅₀ = 2.1 mM); triangles, bridged P^k dimer 2 and SLT-I assay (IC₅₀ = 55 μM); solid circles, STARFISH 3 and SLT-I assay (IC₅₀ = 0.24 nM). Protocol B: open circles, STARFISH 3 and SLT-I assay (IC₅₀ = 0.4 nM); open squares, STARFISH 3 and SLT-II assay (IC₅₀ = 6 nM).

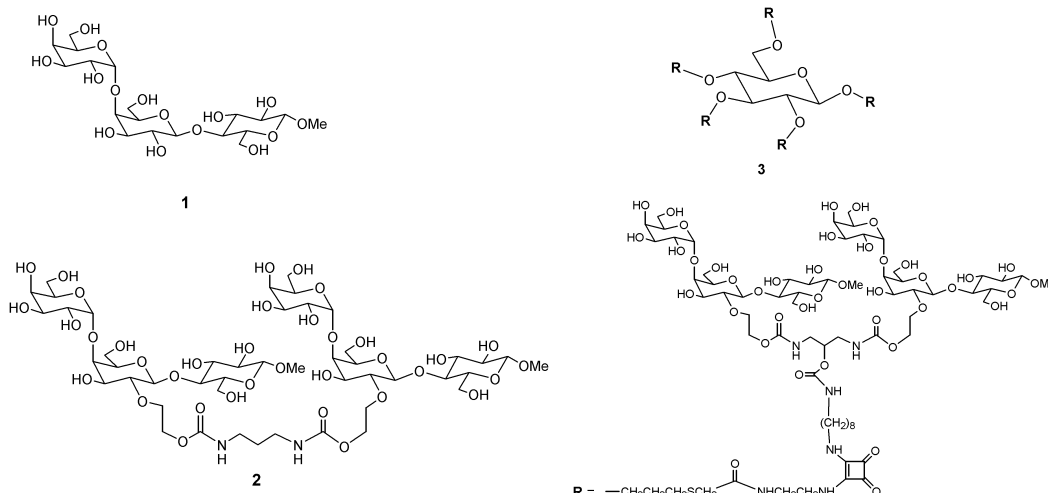


Figure 1 Structures of monomeric and oligomeric P^k inhibitors of Shiga-like toxins. 1, P^k trisaccharide methyl glycoside; 2, dimeric bridged P^k trisaccharide; 3, decameric STARFISH.

B-subunit pentamer^{2,14} thereby allowing all 15 sites to engage cell-surface receptors.

The free energy of binding for the interaction of the B-subunit pentamer with the methyl glycoside of the P^k trisaccharide (compound 1, Fig. 1) is a modest 3.6 kcal mol⁻¹ corresponding to a millimolar dissociation constant (K_d)¹⁵. Functional group modifications of the oligosaccharide's hydroxyl groups showed no substantial gain in binding energy¹⁶, and further investigation of synthetic modifications convinced us that design of a univalent inhibitor based upon functional group modification was unlikely to provide significant affinity increases. 'Random' multivalency of the type seen in acrylamide polymers or in dendrimers has rarely raised lectin-sugar avidity more than 1,000-fold, and the underlying reasons for activity gains of this magnitude are poorly understood⁵. This approach seemed unlikely to provide the 9–10 kcal mol⁻¹ of free energy required to create a SLT inhibitor with nanomolar activity.

Oligovalency 'tailored' to the structure of the B-subunit pentamer offered the best opportunity to design higher-affinity inhibitors, as 15 binding sites are arranged symmetrically across the toxin surface that engages the cell membrane. Binding and cytotoxicity studies on site-directed mutants suggest that the two peripheral binding sites, 1 and 2, are more important than site 3, which is close to the centre of the pentamer. The most striking effects are observed with a site 2 mutant¹⁷. An NMR structure of SLT-I with and without bound ligand determined that site 2 was the most important binding site for soluble trisaccharide¹⁸. We designed a tethered divalent ligand

that could occupy sites 1 and 2 of a single B subunit and perhaps also increase affinity through fortuitous tether-protein contacts. We found the optimum point for tethering ligands that occupy these sites by examining the crystal structure of the bound ligands in each site. Although hydrogen bonds between the protein and sugar hydroxyl groups are numerous, the orientation of the P^k trisaccharide in both sites exposes the hydroxyl group at the C2 position of the central β -galactose residue to solvent and away from the protein surface. At their closest point of separation, the O2' atoms of the Gb₃ ligands in sites 1 and 2 are separated by 9–10 Å. The synthetic assembly of two P^k ligands tethered at the same attachment point, therefore, offered a synthetically conservative approach to a divalent ligand. A simple synthetic route was designed to create a tethered dimer of P^k trisaccharides (compound 2, Fig. 1).

In assays to measure the ability of inhibitors to block binding of a biotin-labelled P^k-BSA (bovine serum albumin) glycoconjugate to SLT-I, compound 2, a bridged P^k trisaccharide dimer (Fig. 2), reached a K_d of 10⁻⁵ M. Although 40-fold higher than the univalent P^k trisaccharide 1, the dimer activity compares poorly with the estimated affinity (K_d = 10⁻⁹ M) of the B-subunit pentamer for cells that express Gb₃ (ref. 19).

However, this bridged P^k dimer could be used in an optimally tailored pentameric cluster that could embrace the entire toxin surface and provide sufficient ligand copies to saturate all the important binding sites. Building tethers of appropriate lengths between distinct bridged carbohydrate dimers that could reside in binding sites 1 and 2 of B-subunit monomers would result in a

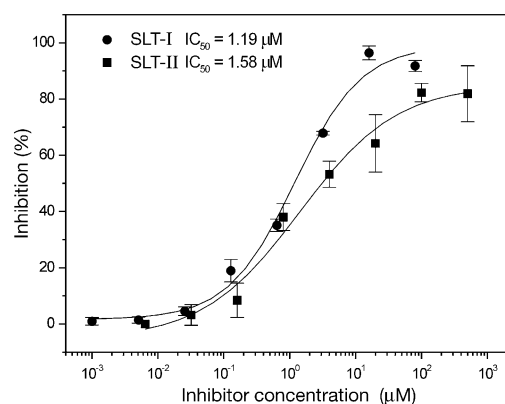


Figure 3 Cytotoxicity assay. Vero cells were incubated with synthetic inhibitors and an otherwise lethal dose of SLT-I or SLT-II. Assays of STARFISH 3 with SLT-I (filled circles) and SLT-II (filled squares).

Table 1 Crystallographic data

Space group	C2
Unit cell	$a = 104.47 \text{ \AA}, b = 71.61 \text{ \AA}, c = 56.36 \text{ \AA}, \beta = 109.0^\circ$
Resolution	2.23 Å
Number of reflections	19,159
R_{meas}	
Overall	16.1%
Highest resolution bin	33.9% (2.23–2.34 Å)
Data completeness	
Overall	99.0%
Highest resolution bin	94.9% (2.23–2.34 Å)
Number of non-hydrogen atoms	
Protein	2,700
STARFISH	240
Solvent	80
R factor	17.1%
R_{free}	18.4%
R.m.s. deviation bond angles	1.251°
R.m.s. deviation bond lengths	0.091 Å

* R_{free} set was selected in shells and consisted of 1064 reflections.

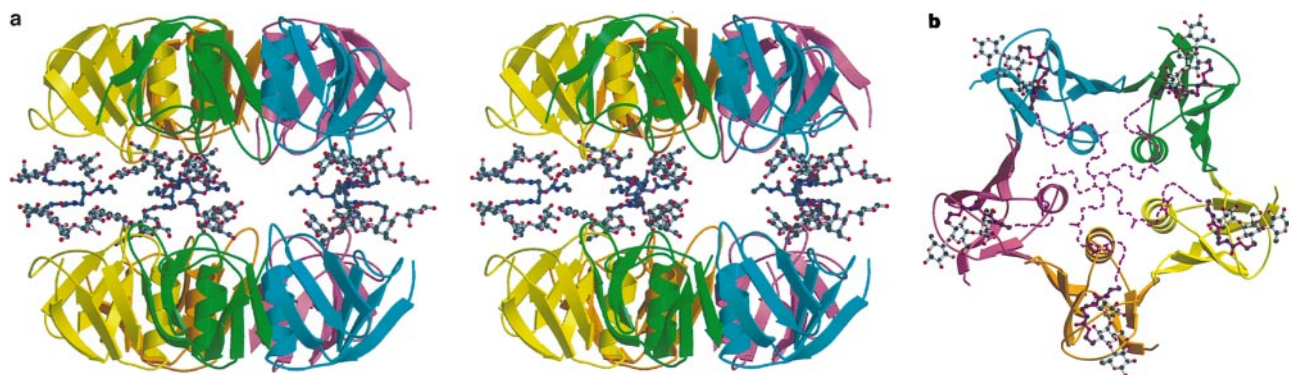


Figure 4 Mode of binding in the STARFISH-SLT-1 complex. **a**, Stereo diagram of the crystallographic dimer of B-subunit pentamers of SLT-I linked by the STARFISH ligand. For the protein, β -strands are illustrated as broad arrows and α -helices as coiled ribbons. The ligand is shown in a ball-and-stick representation, with grey bonds connecting atoms of the carbohydrate component and magenta bonds connecting atoms of the linker.

b, Diagram of half of the STARFISH:SLT-1 B₅ sandwich. The representation is as in **a**, except that dashed magenta lines show a possible conformation for the central component of the linker, which could not be seen clearly in the electron density. These figures were drawn with MolScript²⁹ and rendered with Raster3D³⁰.

multivalent inhibitor that should produce significant avidity gains through entropic savings. Because the peripheral trisaccharides are closer to each other (~ 10 Å) than their respective spacing from the centre of the pentameric complex (~ 30 Å), they can be tethered together and then the dimers assembled into a five-ray cluster (Fig. 1).

We reasoned that pentameric display of P^k trisaccharides at the tips of five appropriately oriented arms that radiate from a central core would allow all five bridged P^k dimers to be simultaneously engaged by the B-subunit pentamer in much the same way that the membrane surface binds toxin. To achieve this display of tethered ligands, a glucose molecule was chosen as the central core²⁰. By derivatizing the glucose as a penta-allyl ether, free-radical reaction with thioglycolic acid was possible and subsequent chain elongation of the radial arms could be achieved by simple uniform chemistry that allowed rapid assembly. Each arm was designed to span the ~ 30 Å from the central pore of the toxin and the tether was designed to bridge binding sites 1 and 2 on each B subunit. The resulting molecule resembles a starfish and hence is named STARFISH (compound 3, Fig. 1).

STARFISH exhibited more than a million-fold increase in inhibition over P^k trisaccharide 1. This subnanomolar activity lies within the range desired for an anti-adhesive therapeutic. Owing to the assay format and lower intrinsic affinities of the SLT-II binding sites, this activity could be measured for SLT-I but not for SLT-II. Protocol A (Fig. 2) uses toxin adsorbed to microtitre plates, and adsorbed SLT-II did not give a signal with the P^k -BSA glycoconjugate reporter molecule because the lower intrinsic affinities of the SLT-II binding sites result in a short half-life of the bound enzyme-linked immunosorbent assay (ELISA) reporter molecule which dissociates from the plate during the multiple washing steps.

To assay SLT-II binding, we developed a solid-phase inhibition assay that allowed the comparison of SLT-I and SLT-II in a single assay format (protocol B, Fig. 2). A synthetic P^k -like glycolipid, developed for detecting SLTs with a biosensor²¹, was used as the solid-phase capture ligand, and bound toxin was detected by a toxin-specific antibody. In this format, both SLT-I and SLT-II bound to the plate with SLT-II showing ~ 10 -fold weaker avidity for the immobilized ligand. Again, the STARFISH inhibitor had a half-maximal inhibitory concentration (IC_{50}) of 4×10^{-10} M with SLT-I, but also had an IC_{50} of 6×10^{-9} M with SLT-II (Fig. 2). The assay thus confirmed that the ligand for SLT-II is the P^k trisaccharide.

The ability of inhibitors to protect host cells against a lethal dose of SLT-I in culture can be determined using Vero cells¹¹. STARFISH inhibitor provided effective protection of Vero cells cultured in the presence of SLT-I and SLT-II, even over the 2-day co-incubation period (Fig. 3).

Crystallographic study of the complex formed between the B-subunit pentamer of SLT-I and STARFISH revealed a mode of binding that differs from the one originally envisaged by 'rational design'. The crystal structure shows that one STARFISH molecule binds to not one but two B-subunit pentamers (Fig. 4). Instead of bridging sites 1 and 2, the tethered P^k trisaccharides of STARFISH bind to two B-subunit monomers from separate toxin molecules. The two B-subunit pentamers are related by a crystallographic twofold axis that passes through the pseudo-twofold symmetric STARFISH molecules, and only site 2 is occupied in all the B-subunit monomers. The trisaccharide is well ordered (mean B-factors: α -galactose residues, 21.5 Å²; β -galactose residues, 28.9 Å²; glucose residues, 43.6 Å²), its binding interactions are identical to those seen for the univalent ligand⁶, and STARFISH binding does not perturb the protein. As, in this experiment, the concentration of STARFISH was sufficient to form a 1:1 complex, the formation of the 2:1 sandwich must be thermodynamically favoured. Table 1 gives a summary of the crystallographic statistics. The design elements that target the inhibitor to embrace the toxin surface clearly succeeded in placing the P^k trisaccharides in each B subunit.

However, the bivalent ligand at the end of each arm occupies only site 2 of a single B subunit and does not bridge sites 1 and 2 as planned. This results in an unexpected benefit as each arm now presents an unbound P^k trisaccharide and these five epitopes are fortuitously spaced to bind across the face of a second toxin molecule. This interaction is favoured over intramolecular bridging of sites 1 and 2, owing presumably to the previously reported higher affinity of binding site 2 and perhaps also to potential strain in the bridge.

The high inhibitory activity of a multivalent cluster tailored to complement the distribution of oligosaccharide-binding sites across the toxin surface is unusual for glycoconjugates of such size and modest valency. The efficacy of this particular design might be improved by decreasing the entropic penalty through the use of more rigid spacers that link the distal epitopes to the central core. Perhaps the most important feature of this design is the use of dimers that bridge two toxin molecules. Intriguingly, the formation of a toxin dimer indicates that the general inhibitor design may be applicable to other bacterial AB₅ toxins such as cholera toxin or heat-labile enterotoxin. Specific complexation of STARFISH with site 2 of SLT-I, which also possesses the highest affinity in solution^{17,18}, is significant as the single sugar-binding site per B subunit that is found in cholera and heat-labile toxins² corresponds topologically to site 2 of SLT⁶. Hence, the STARFISH bridged-dimer design feature may be general and capable of extension to cholera toxin and heat-labile toxin. In such cases, where each B subunit contains only one sugar-binding site, the ability to bridge between toxin molecules would be essential to achieve potent inhibitory activity.

We have shown that the STARFISH inhibitor protects susceptible cells against prolonged exposure not only to SLT-I but also to the reportedly more clinically significant SLT-II (ref. 22), a toxin for which there were previously no inhibitors. In view of their potent activity, these inhibitors represent a potential treatment for preventing kidney damage that occurs after serious cases of toxin poisoning. This confirms the potential of carbohydrates as new and viable bacterial anti-adhesive therapeutics, which was first suggested by Sharon and co-workers⁴ some 20 years ago. □

Methods

Inhibitors

The inhibitors were synthesized by methods that will be published elsewhere. We characterized them by elemental analysis, NMR, and MALDI (matrix-assisted laser desorption/ionization), electrospray and ion cyclotron resonance mass spectrometry to confirm their structures. Shiga-like toxins I and II were purified according to published methods²³.

Solid-phase binding assays

Protocol A: SLT-I dissolved in PBS (100 μ l; 2.5 μ g ml⁻¹) was coated on 96-well ELISA plates (18 h at 4 °C). The plate was washed five times with PBST (PBS containing Tween 20, 0.05% v/v), blocked for 1 h with milk (DIFCO, 2.5% in PBS, 100 μ l). The plate was washed twice with PBST. A P^k trisaccharide conjugated to BSA and biotinylated (10 μ g ml⁻¹) was mixed with inhibitor at concentrations in the range 0.1 nM to 10 mM, and the mixture (100 μ l) was added to the coated microtitre plate and incubated at room temperature (18 h). The plate was washed five times with PBST and streptavidin horseradish peroxidase conjugate (100 μ l) was added and incubated for 1 h at room temperature. The plate was washed five times with PBST, 3,3',5,5'-tetramethylbenzidine (TMB, 100 μ l) was added, and after 2 min the colour reaction was stopped by the addition of 1 M phosphoric acid (100 μ l). Absorbance was measured at 450 nm and the percentage inhibition was calculated using wells containing no inhibitor as the reference point.

Protocol B: a synthetic P^k trisaccharide attached to a C₁₆ aglycon terminated by an ω -thiol and oxidized to the corresponding disulphide 4 (ref. 22) was dissolved in PBS (10 μ g ml), and coated on 96-well ELISA plates (100 μ l, 18 h at 4 °C). The plate was washed five times with PBST and blocked for 1 h at room temperature by incubation with 1% BSA in PBS (100 μ l). The plate was washed three times with PBST, and SLT solution (100 μ l) was added to the plate (SLT-I at 0.05 μ g ml⁻¹ or SLT-II at 0.1 μ g ml⁻¹). The SLT solution with or without inhibitor was incubated for 18 h at room temperature. The plate was washed five times with PBST, and rabbit anti-SLT-I or SLT-II solution (100 μ l) diluted in PBS (1:1000) was incubated for 1 h at room temperature. After the plate had been washed five times with PBST, commercial horseradish-peroxidase-labelled, goat anti-rabbit antibody solution (100 μ l) diluted (1:2000) in PBS was incubated for 1 h at room temperature. The plate was washed five times with PBST, and colour was developed and measured as described in protocol A.

Cytotoxicity assay^{11,23}

STARFISH inhibitor was dissolved in double-distilled, deionized water. Stock solutions of purified SLT-I and SLT-II were prepared at concentrations of 400 ng ml⁻¹ and 2 µg ml⁻¹, respectively, in unsupplemented MEM. Serial dilutions, in unsupplemented MEM, of inhibitor solution were prepared using a 96-well microtitre plate. Next, 5 µl of stock SLT-I or SLT-II solution was added to each well (to 80 µl final volume) of the appropriate rows in the dilution plate. The solution in each of the dilution plate wells was thoroughly mixed and the microtitre plate was incubated for 1 h at 37 °C, after which 20 µl from each well was transferred to the corresponding well of a 96-well microtitre plate containing confluent Vero cell monolayers and 200 µl of MEM supplemented with fetal bovine serum. The Vero cell microtitre plate was incubated for an additional 48 h in a 37 °C incubator in an atmosphere of 5% CO₂/95% air. The Vero cell monolayers were then fixed with methanol and cytotoxicity was measured as described¹¹.

Crystallography

A solution of the complex was made by adding 15 µl of a solution of STARFISH (0.35 mM in water) slowly to 15 µl of SLT-I B subunit (10 mg ml⁻¹, 0.1 M NaCl and 10 mM Tris pH 8.0) with agitation. We mixed this solution with an equal volume of reservoir solution (28% saturated ammonium sulfate, 2% 2-methyl-2,4-pentanediol, 0.1 M NaCl and 0.1 M Hepes pH 7.0) for hanging drops. Crystals grew at room temperature as clusters of needles. Diffraction data, collected on a MAR345 detector mounted on a Rigaku rotating anode generator, extended to 2.23 Å resolution (Table 1). A molecular replacement solution was obtained in AMoRe²⁴ using the B subunit of the crystal structure of the SLT-I B-subunit pentamer complexed with Gb₃ (ref. 6) as a model. The test set was selected in thin shells to limit the bias that may be introduced by the fivefold non-crystallographic symmetry (NCS) present in the asymmetric unit. Refinement was carried out in CNS²⁵ using maximum-likelihood targets²⁶, and strict NCS followed by restrained NCS. Manual intervention in between cycles of refinement using the XTALVIEW package²⁷ and averaged cross-validated SigmaA²⁸ maps allowed the building of part of the STARFISH molecule. The STARFISH molecule is fully occupied in the crystal, but, because of symmetry, parts of the linker are only half occupied in the crystallographic asymmetric unit.

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Correspondence and requests for materials should be addressed to D.R.B. Coordinates and structure factors have been deposited in the Protein Data Bank under accession code 1qnu.

How self-tolerance and the immunosuppressive drug FK506 prevent B-cell mitogenesis

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Therapy for transplant rejection, autoimmune disease and allergy must target mature lymphocytes that have escaped censoring during their development. FK506 and cyclosporin are immunosuppressants which block three antigen-receptor signalling pathways (NFAT, NFκB and JNK), through inhibition of calcineurin¹, and inhibit mature lymphocyte proliferation to antigen^{2–4}. Neither drug induces long-lived tolerance *in vivo*, however, necessitating chronic use with adverse side effects. Physiological mechanisms of peripheral tolerance to self-antigens provide an opportunity to emulate these processes pharmacologically. Here we use gene-expression arrays to provide a molecular explanation for the loss of mitogenic response in peripheral B-cell anergy, one aspect of immunological tolerance⁵. Self-antigen induces a set of genes that includes negative regulators of signalling and transcription but not genes that promote proliferation. FK506 interferes with calcium-dependent components of the tolerance response and blocks an unexpectedly small fraction of the activation response. Many genes that were not previously connected to self-tolerance are revealed, and our findings provide a molecular fingerprint for the development of improved immunosuppressants that prevent lymphocyte activation without blocking peripheral tolerance.

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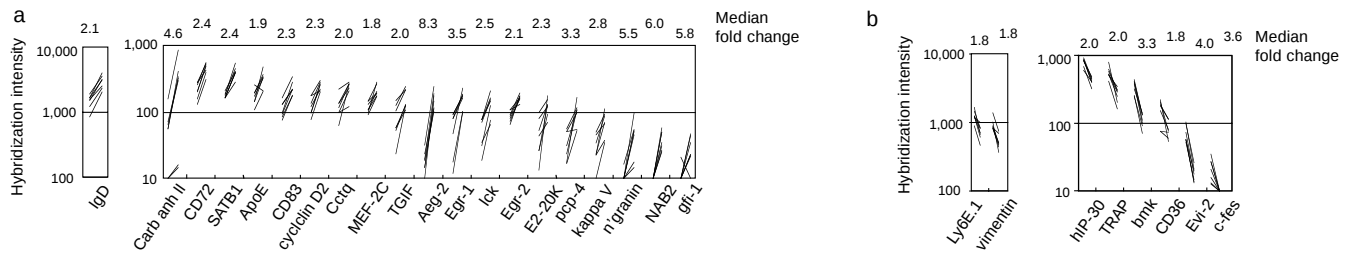


Figure 1 Tolerance response to self-antigen, $P < 0.00034$. **a**, Upregulated genes. Each line represents samples from each genotype prepared in parallel on the same day. The left end of each line represents hybridization intensity in naive cells; the right end represents tolerant cells. Five sets of data are from negatively depleted B-cell preparations; two are

from FACS-purified cells. For one experiment, two lines connect a single naive cell sample with two tolerant cell samples. Known concentrations of spiked bacterial transcripts allow an approximate calibration of 5 intensity units per copy per cell ($\pm$ twofold¹⁰) assuming 300,000 transcripts per cell. **b**, Downregulated genes, represented as in **a**.

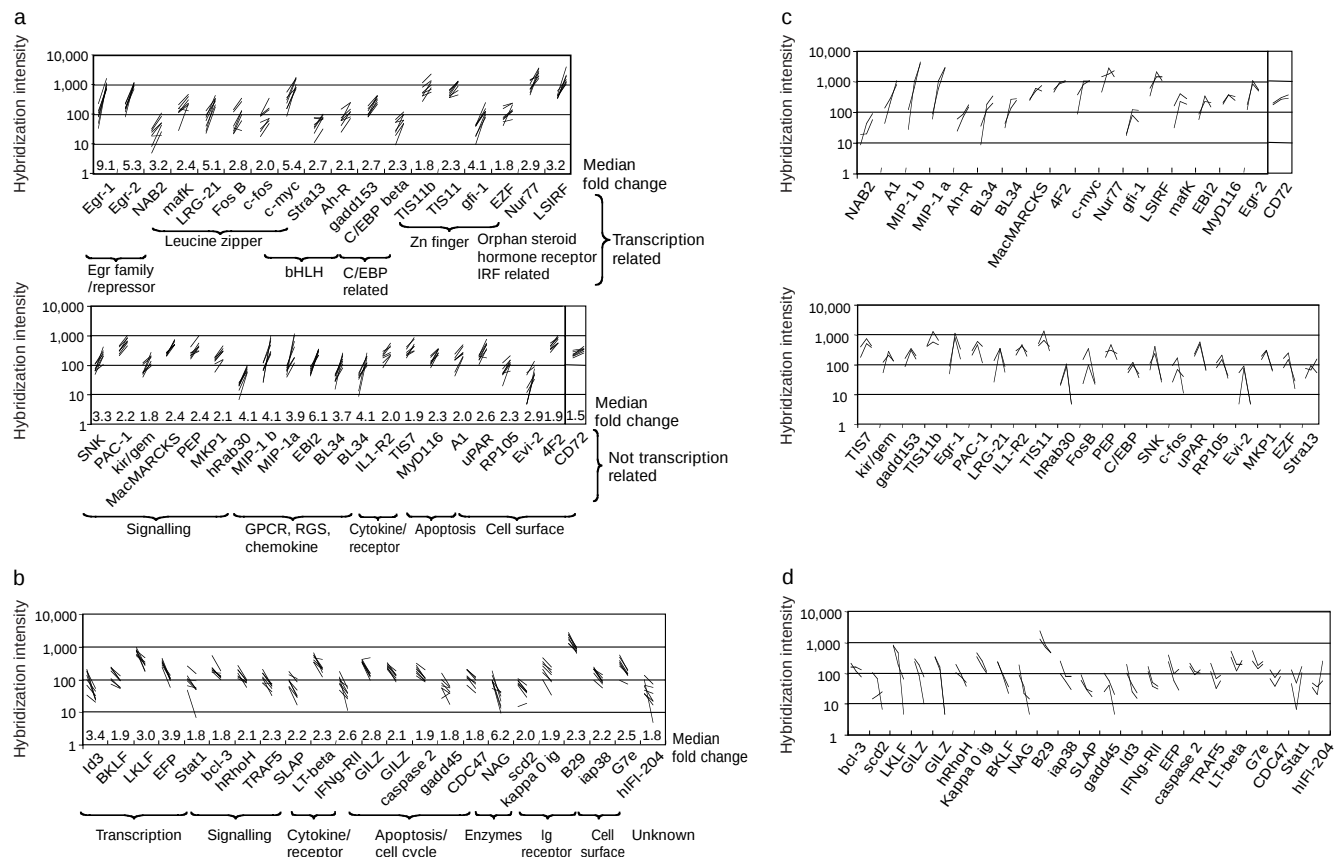


Figure 2 Activation response to foreign antigen, $P < 0.00018$. **a**, Upregulated genes, 1 h activation, 7 experiments, each line represents 1 experiment. The left end of the line represents mock-stimulated cells; the right end represents Ig^{HEL} cells stimulated with HEL (3 experiments) or non-transgenic cells stimulated with anti- μ (4 experiments). Responses of Ig^{HEL} and non-transgenic B cells were essentially identical (see also Supplementary Information). CD72 is shown but only increased 1.5-fold. BL34 (RGS1) is

represented twice on the arrays; both sets of data are shown. **b**, Downregulated genes, 1 h activation, 2 experiments, represented as in **a**. **c**, Upregulated genes, 1 and 6 h activation, 2 experiments, HEL stimulation of Ig^{HEL} transgenic B cells. The left end of the line represents 1 h mock stimulation, the middle of the line 1 h activation and the right end of the line 6 h activation. Genes are in order of exaggerated, sustained and transient increases. **d**, Downregulated genes, 1 and 6 h activation, represented as in **c**.

Homogeneous populations of naive B lymphocytes specific for a well-defined antigen, hen egg lysozyme (HEL), were obtained from immunoglobulin transgenic mice (Ig^{HEL} mice). Tolerant B cells carrying the same Ig^{HEL} receptor and matched for developmental stage were obtained from double transgenic mice that also express soluble HEL as a neo-self-antigen (sHEL:Ig^{HEL} mice). Unlike naive cells, tolerant cells fail to proliferate or make antibody to HEL *in vitro* or *in vivo*^{5,6}. Instead, repeated stimulation by self-HEL causes them to make responses that actively reinforce tolerance, such as altered migration, and Fas-dependent and Fas-independent apoptosis⁵. These responses collectively describe a state of anergy and are maintained by partial signalling downstream of the antigen

receptor^{5,7,8}. Tolerant B cells in the spleen of sHEL:Ig^{HEL} mice have been exposed to self-antigen from their development in the bone marrow throughout their $\sim$ 1-week life span⁵. They appear homogeneous with respect to the aspects of anergy described above, and as measured in single cells by continuous calcium oscillations⁷ and uniformly low expression of the activation markers B7-2 (ref. 9) and CD69.

We first screened for gene-expression changes in tolerant B cells using Affymetrix oligonucleotide expression arrays to monitor 6,500 genes and expressed sequence tags (ESTs) (reviewed in ref. 10). For exemplar accession numbers, raw data and statistical methods, see Supplementary Information. We compared expression

between five tolerant B-cell preparations and four naive B-cell preparations; paired samples were purified by negative selection with magnetic beads. A further two preparations of each were purified by positive selection on a cell sorter. Using an algorithm that requires consistency between both purification methods, expression of only 20 genes was significantly increased and that of 8 genes was significantly decreased in tolerant cells (Fig. 1, $P < 0.00034$). Increased messenger RNA for the light chain V κ 5 segment is consistent with the increased frequency of variant B cells that have rearranged endogenous immunoglobulin genes in sHE-L:Ig^{HEL} mice. Increased expression of IgD and Egr-1 in tolerant B cells was confirmed by western blotting, and increased expression of CD72 has been confirmed by flow cytometry (J. Parnes, personal

communication). Aside from Egr-1, none of these genes have previously been connected to self-tolerance.

In parallel, we analysed the same set of 6,500 genes for expression changes in naive Ig^{HEL} B cells during a well-defined system of synchronous activation by HEL *in vitro* (Fig. 2). In 7 independent replicate experiments, mRNAs for only 37 genes were significantly increased and 22 were decreased by 1-hour activation, when compared with control cells cultured in parallel without antigenic stimulus ($P < 0.00018$). Many of these 59 transcripts encode transcriptional regulators. Although a small number have been previously identified as early response genes in B cells^{11–17}, which validates our data, most were not previously known to participate in B-cell activation responses.

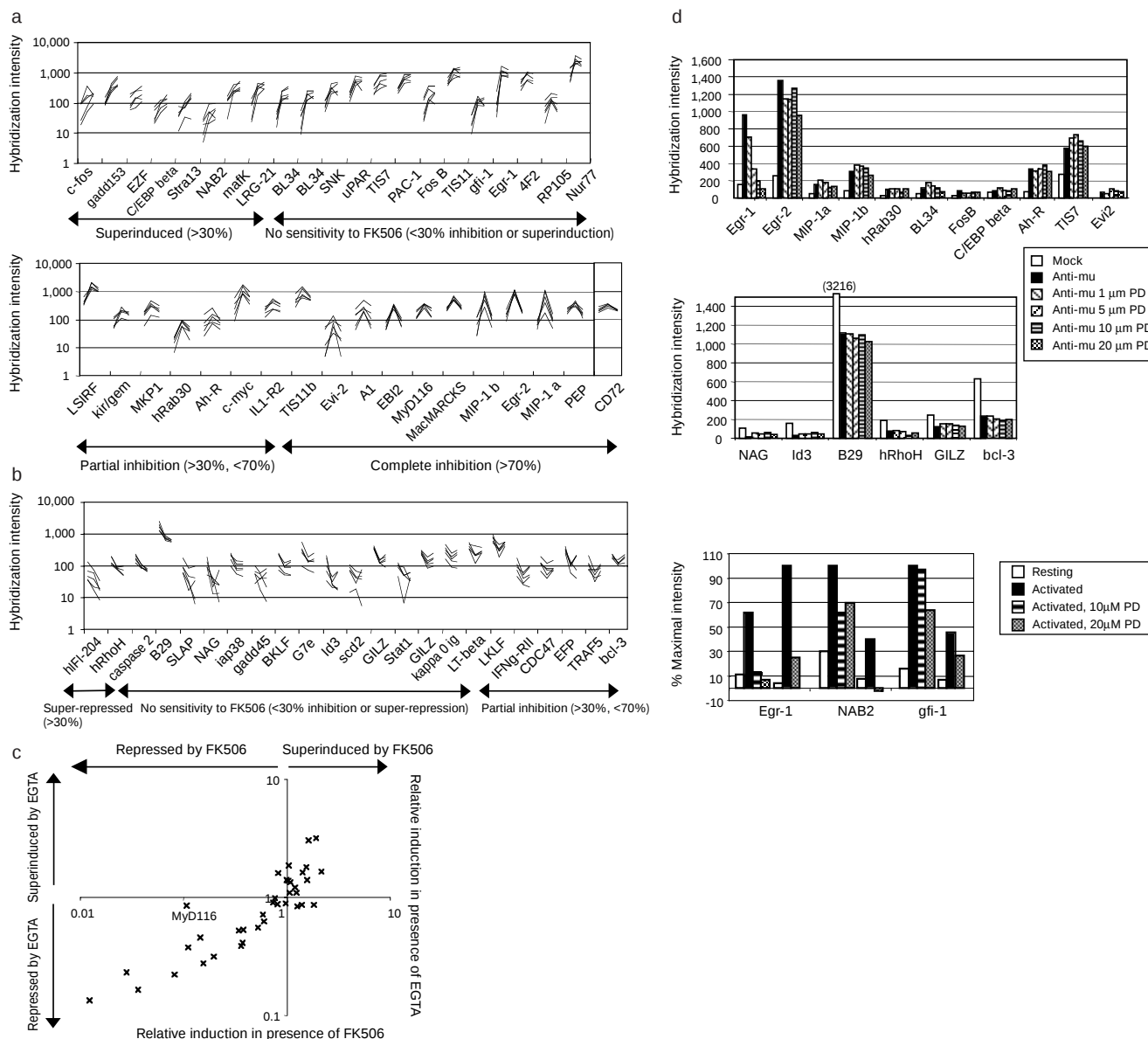


Figure 3 Effect of FK506, EGTA and PD98059 on activation response. **a**, FK506 sensitivity, upregulated genes; 5 experiments and genes are shown in increasing order of median FK506 sensitivity. The left end of the line represents mock stimulation, the middle of the line represents 1 h activation, and the right end of the line represents 1 h activation in the presence of FK506. Three experiments are Ig^{HEL} cells stimulated with HEL with or without FK506; two experiments are non-transgenic cells stimulated with anti-mu with or without FK506. **b**, FK506 sensitivity of downregulated genes, represented as in **a**. **c**, EGTA compared with FK506. For 1 h upregulated genes (Fig. 2a), relative induction in the presence of EGTA was calculated as average (antigen/EGTA – mock)/(antigen – mock)

of 2 experiments of Ig^{HEL} cells stimulated with HEL with or without EGTA. Relative induction in the presence of FK506 was calculated as median of (antigen/FK506 – mock)/(antigen – mock) over five experiments. An outlier, MyD116, is labelled. **d**, Effect of PD98059. Upper two panels, regulation of 17 1-hour response genes in the presence of various concentrations of PD98059 (PD). Lower panel, three transcripts upregulated by both foreign and self-antigen are sensitive to PD98059. First four columns, non-transgenic B cells stimulated with anti-mu for each gene; last three columns, Ig^{HEL} transgenic B cells stimulated with HEL.

Gene expression was much more extensively altered after 6 hours of activation. Although mRNAs for many of the 1-hour-induced genes had decreased by this time (for example, *Egr-1*, *Pac-1*, *c-fos*, *Fos B*; Fig. 2e), others in the early response set showed sustained or exaggerated responses at 6 hours (for example, *A1* and *MIP-1a/b*; Fig. 2c; *LKLF* and *GILZ*, Fig. 2d). About 300 additional genes had increased mRNA and 200 had decreased mRNA at this time, consistent with movement to G1 phase of cell cycle, including upregulation of *CDK4* and *cyclin D2* (ref. 18).

Comparison of the activation response with tolerance showed that most of the transcripts regulated by self-antigen are also regulated after 1 or 6 hours of activation. For a detailed comparison, including analysis of the effects of tissue culture, see Supplementary Information. Shared transcript changes include *NAB2* and *neurogranin*, which were comparably upregulated in both types of response, and *Egr-1*, *Egr-2*, *Gfi-1*, *cyclin D2* and *Cctq*, which were more highly induced during activation. A subset of the tolerance response genes was not regulated after activation, including *Aeg-2*/*Crisp-3*, a secreted molecule encoded in the MHC and regulated by *Oct-2* (ref. 19). Conversely, the activation response was almost completely blocked in tolerance: only 16 out of more than 500 transcript changes caused by antigen in 6 hours of activation were also regulated by self-antigen in tolerant cells ($P < 0.05$, fold change

>1.8 , at least 1 out of 2 experiments with sorted cells).

In the B-cell activation cultures, we stimulated a third group of cells in the presence of a clinically relevant concentration of FK506 to determine how well immunosuppression mirrors the molecular effects of B-cell anergy. Out of the 59 transcript changes detected 1 hour after B-lymphocyte activation, only one-third were efficiently suppressed by this dose of FK506 (Fig. 3). Some early response genes (for example, *gadd153*) were superinduced. As FK506 affects transcript profiles in yeast independently of calcineurin²⁰, we tested whether chelation of extracellular calcium had a similar effect on suppression of B-cell activation. Activation of naive B cells in the presence of EGTA reproduced the effects of FK506 (Fig. 3c), confirming that FK506 affected transcript levels through a calcium/calcineurin-dependent pathway. We did not note any significant FK506-induced changes in transcripts other than those altered in the antigen activation response.

Transcript changes in B-cell activation that are suppressed by FK506 and EGTA can be assigned downstream of calcium/calcineurin, and further subdivided on the basis of their expression in tolerant cells (Fig. 4a). The low calcium oscillations in tolerant cells are sufficient to induce nuclear translocation of NFAT but not to activate NFkB nor JNK^{7,21}, although all three pathways are dependent on calcium/calcineurin. Thus, FK506-sensitive upregulation of genes not altered in tolerant cells implies signalling through NFkB or JNK (for example, *A1*, *MIP1*, *LSIRF*, *c-myc*), whereas FK506-sensitive genes that are also upregulated in tolerance would be expected to be downstream of NFAT (for example, *Egr-2* and *CD72*). Upregulation of *A1* has recently been described²² as downstream of *c-rel*, which is consistent with our data.

The ERK pathway is also activated in B-cell anergy and activation. To determine downstream transcriptional effects of ERK, we titrated the MEK inhibitor PD98059 (ref. 23) in a B-cell activation experiment. Upregulation of *Egr-1* was totally inhibited by 20 μ M PD98059 and was 50% inhibited by 5–10 μ M, consistent with the potency of PD98059 against recombinant MEK²³ (Fig. 3d). Regulation of other early response genes was less sensitive than *Egr-1*. Induction of three transcripts (*Egr-1*, *NAB2* and *Gfi-1*) that are upregulated by both self-antigen and foreign antigen was sensitive to PD98059 (Fig. 3d) but insensitive to FK506 (Fig. 3a). Continuous activation of the ERK pathway by self-antigen thus appears to have distinct transcriptional consequences (Fig. 4a).

Our findings provide the first molecular explanation for how self-tolerance prevents lymphocyte mitogenesis (Fig. 4b). Given the continuous signalling through the ERK and NFAT pathways in response to self-antigen⁷, it is remarkable how few mitogenic response genes are triggered in anergic B cells. The loss of mitotic response to antigen is explained by failure to upregulate *LSIRF*, a B-cell myeloma protooncogene²⁴ that is an essential transcription factor for B-cell mitogenic responses²⁵, and failure to upregulate *A1*, an anti-apoptotic protein that is sufficient and apparently necessary to block apoptotic responses to antigen in B cells²². The block in induction of the B-cell lymphoma protooncogene, *c-myc*, is also likely to contribute because increased expression of *c-myc* is sufficient to promote B-cell blastogenesis in transgenic mice²⁶. Likewise, tolerant B cells lacked any downregulation of the inhibitory transcription factors *LKLF* and *BKLF*. A decrease in levels of *LKLF* may be an obligatory, although apparently not a sufficient, step in B-cell activation as T cells that are deficient in *LKLF* have a spontaneously activated cell-surface phenotype²⁷. By comparison, it is surprising how little of the early mitogenic response is suppressed by FK506 given its ability to block foreign-antigen-stimulated NFAT, NFkB and JNK⁷. Inhibition of *A1* induction by FK506 may alone be sufficient to explain the anti-mitogenic effects of FK506, as activation in the presence of FK506 is associated with increased B-cell death⁴. It will be interesting in future studies to examine how the activation gene pattern shifts to the tolerance pattern when naive B cells are induced to become anergic over several days of continuous

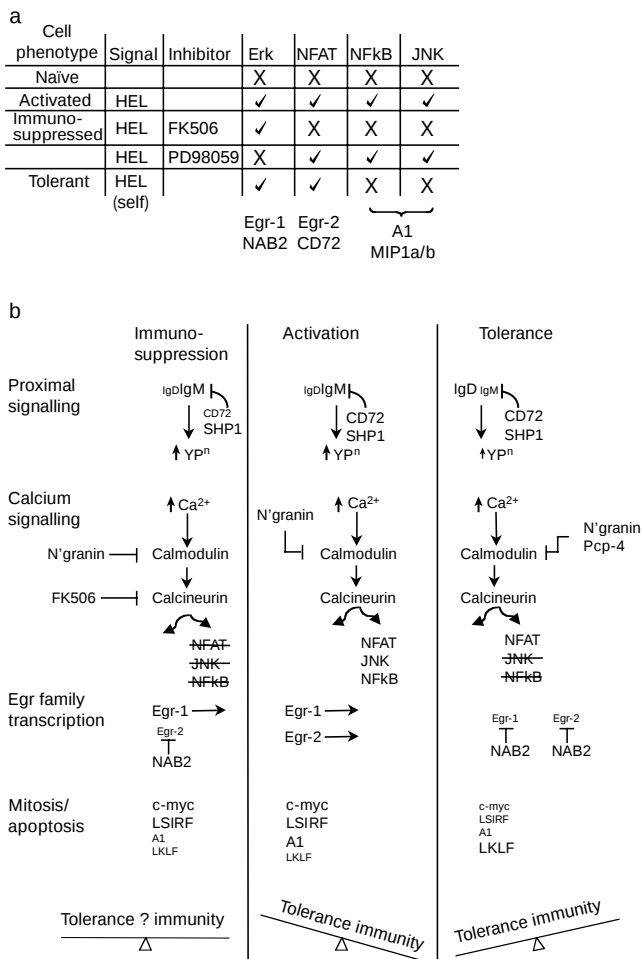


Figure 4 Expression profiles connect genes to pathways and immune responses. **a**, Summary table of biochemical pathways in tolerant and naive cells exposed to foreign antigen in the presence or absence of FK506 and PD98059. **b**, Summary of key differences in response to antigen during tolerance, activation and immunosuppression. Font size reflects mRNA or protein expression level relative to mock-stimulated cells (immunosuppression and activation panels) or naive cells (tolerance panel). N' granin, neurogranin; Y^P, tyrosine phosphorylation.

exposure to antigen^{5,8}.

Many of the genes associated with the tolerance response might be predicted to have negative regulatory functions for maintaining the tolerant state (Fig. 4b). Egr-1 and Egr-2 are transcription factors implicated in B-cell mitogenesis^{11,12}, but their mitogenic activity is likely to be repressed in tolerant cells by relatively high expression of NAB2, an Egr family inhibitor²⁸. Neurogranin and pcp-4 inhibit calmodulin²⁹ and may regulate the downstream effects of calcium oscillations in tolerant cells^{7,21}. IgD represents the primary receptor isotype on tolerant B cells, through which repeated binding of self-antigen triggers low calcium oscillations. IgD signalling may be decreased relative to naive cells⁶ by increased levels of CD72, which recruits the inhibitory tyrosine phosphatase SHP-1 (ref. 30) (Fig. 4b).

Inhibition of Egr-2 and CD72 by FK506 shows that this drug interferes with components of the active self-tolerance response, which may limit its efficacy in establishing or restoring tolerance in autoimmunity and transplantation. To develop more effective drugs, the unique transcript signature in anergic cells could be used in high-throughput assays as a surrogate marker for tolerance. A desired small molecule would suppress members of the subset of activation-only early response genes defined here, while leaving unaffected the subset of early response genes that also participate in tolerance, so that tolerance would be actively (re-)established. □

Methods

B-cell purification and stimulation

For each cell preparation, splenic B cells were purified and pooled from several mice at room temperature in 1% bovine calf serum in RPMI. The spleen cells were stained with CD4, CD8 and Mac-1 FITC-conjugated antibodies (Caltag) and depleted of T cells and macrophages with sheep anti-FITC magnetic beads (Perseptive Biosystems). The remaining cells were 85–95% B220 positive and were either lysed immediately (naive and tolerant cell preps) or stimulated in RPMI with 1% serum at 37 °C at $(2-3) \times 10^6$ cells ml⁻¹. For stimulation experiments, HEL (Sigma) was used at 500 ng ml⁻¹, PD98059 (NEB) at 20 μM unless stated otherwise, ionomycin (a gift from G. Crabtree) at 1 μM and EGTA at 3 mM. The concentration of FK506 was chosen to be within the range maintained in the blood of kidney and liver transplant patients receiving FK506 (also called Tacrolimus and Prograf; information on dosing from <http://www.fujisawa.com/info/medinfo/mnpginst.htm>). Cells were pre-incubated for 45 min with PD98059, 15 min with FK506 and 2 min with EGTA before addition of HEL or anti-μ. Mock stimulations were carried out by addition of carrier alone for stimuli or inhibitors. At the end of the incubation, the cells were pelleted by centrifugation, resuspended in a minimal volume of medium (~50 μl) by pipetting and lysed in 0.5–1 ml Trizol (Gibco BRL). Naive and tolerant B cells were also purified by cell sorting for B220 positive, CD21^{medium} cells. Marginal zone cells (CD21 high) were excluded from the gate. Between 10^7 and 5×10^7 magnetic-bead-selected B cells, or $(1.5-3) \times 10^6$ cell-sorter-purified B cells were used for RNA preparation.

RNA preparation, array hybridization and data analysis

RNA labelling and array hybridization were done as described¹⁰. For detailed methods and statistical analysis, see Supplementary Information.

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Localizative of apical epithelial determinants by the basolateral PDZ protein Scribble

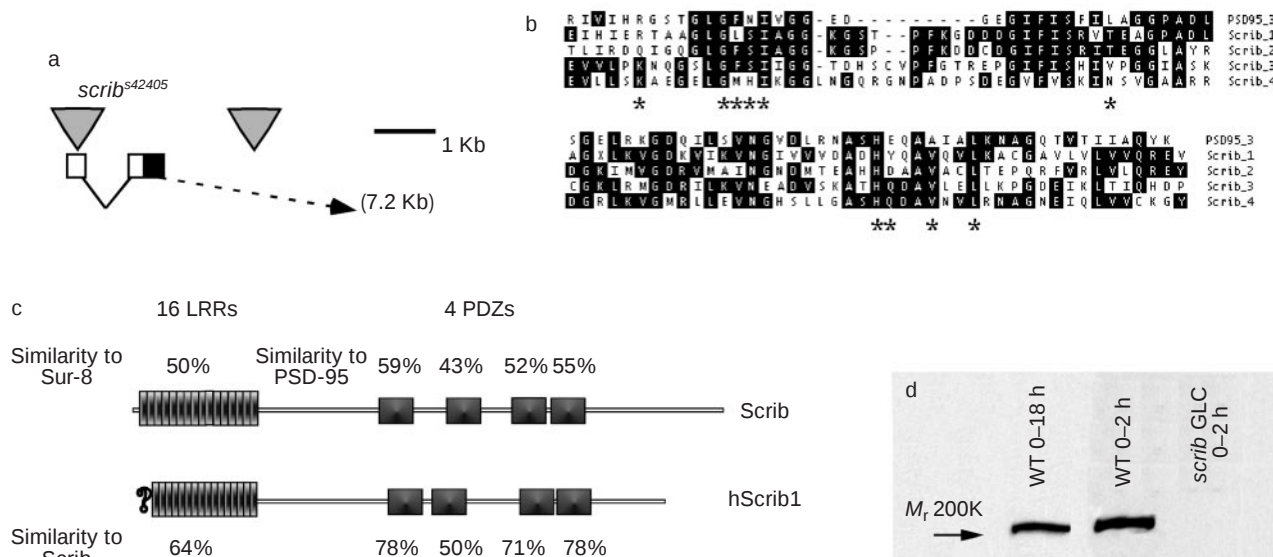
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The generation of membrane domains with distinct protein constituents is a hallmark of cell polarization. In epithelia, segregation of membrane proteins into apical and basolateral compartments is critical for cell morphology, tissue physiology and cell signalling. *Drosophila* proteins that confer apical membrane identity have been found^{1,2}, but the mechanisms that restrict these determinants to the apical cell surface are unknown. Here we show that a laterally localized protein is required for the

To understand the requirement of *scrib* for epithelial organization, we cloned the *scrib* gene (Fig. 2a; see also Methods). Plasmid

Figure 1 *scrib* mutations cause disorganization of epithelia. **a**, Wild-type cuticle. **b**, Cuticle from *scrib* GLC embryo. Only folded strips and whorls of disintegrating cuticle are produced. **c**, Scanning electron micrograph of a wild-type stage-14 embryo. **d**, Similar image of a *scrib* embryo. The highly integrated epithelium of wild-type embryos is falling apart; flattened and rounded cells are seen pulling away from their neighbours. **e**, Confocal microscope image of stage-15 wild-type embryonic epidermis stained for α -Spectrin. Note the regular columnar monolayer organization of the epithelium. **f**, Similar image of a *scrib* embryo. The epidermal cells are rounded, irregularly shaped, and pile up upon each other.



that of PSD-95 PDZ3; consensus identities are shaded in black. Asterisks indicate residues of PSD-95 that are predicted to determine its selectivity for binding an S/TVX consensus sequence at the C terminus of transmembrane proteins. **d**, Western blot of 0–18-hour and 0–2-hour wild-type (WT) embryos, and 0–2 hour *scrib* GLC embryos, probed with the anti-Scrib antibody. The antibody detects a large maternal contribution of Scrib that is absent in the mutant embryos.

includes a Kozak-like consensus sequence followed by a methionine codon at the start of a 5.8-kb open reading frame (ORF). Upon heat-shock induction, the A2 cDNA rescues the lethality of strong heteroallelic *scrib* combinations, showing that it encodes *scrib* function.

The *scrib* ORF is predicted to encode a protein of 1,731 amino acids with a relative molecular mass of 195,000 (M_r 195K). Sequence analysis reveals the presence of two known protein–protein interaction motifs (Fig. 2b). At the amino terminus of Scrib is a set of 16 leucine-rich repeats (LRRs). This region is very similar to the Ras-binding LRRs of the proteins Sur-8 from *Caenorhabditis elegans* and adenylate cyclase from yeast^{4,5}. Distributed throughout the remainder of the protein are four PDZ domains. The PDZ domains of Scrib are highly similar to those of PDZ3 from PSD-95, a human homologue of fly Discs-large (Fig. 2c). Comparison of Scrib residues with those of PSD-95 indicates that all four Scrib PDZ domains are Type 1A, which are predicted to bind to the consensus S/TXV at the carboxy terminus of proteins⁶. Scrib has at least one

human homologue, encoded by the KIAA0147 cDNA⁷, which we call *hscrib1* (Fig. 2b). No obvious signal sequences or transmembrane domains are predicted in either Scrib or *hscrib1* by standard algorithms, suggesting that Scrib is a cytosolic protein. We note, however, that Densin, a protein with 16 LRRs and one PDZ domain, has been suggested to have transmembrane topology²⁹. We cannot exclude the possibility that Scrib is an atypical transmembrane protein.

To determine the subcellular localization of Scrib, we generated antibodies against Scrib peptides (see Methods). Two polyclonal sera recognize a band of ~210K on embryonic western blots. This band is absent in extracts from *scrib* embryos (Fig. 2d). In fixed tissue, Scrib is present at low levels in precellular embryos, where it is found in the actin caps that overlay the still-dividing blastoderm nuclei. As cellularization proceeds during cycle 14, Scrib staining becomes associated with the cell membranes as they invaginate basally towards the centre of the embryo (Fig. 3a). During gastrulation, however, Scrib relocates specifically to a relatively apical position

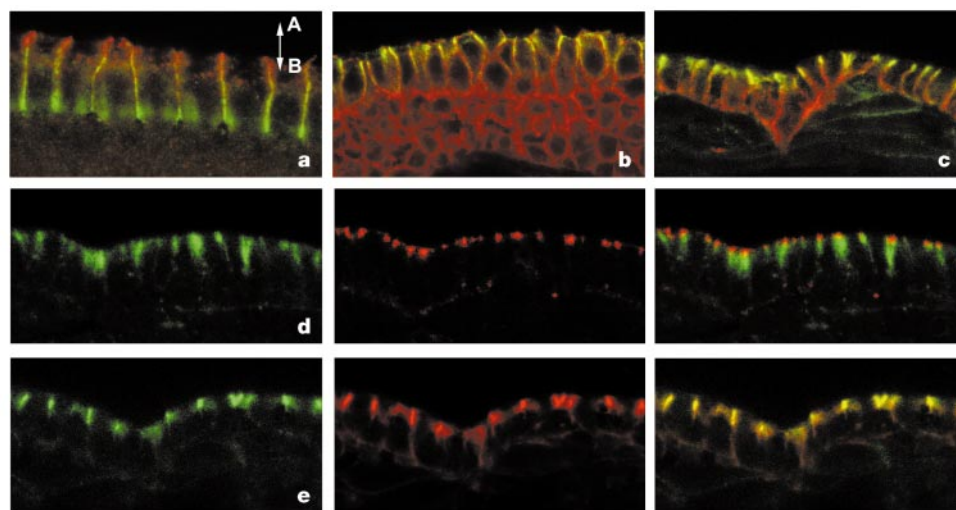


Figure 3 Expression and localization of Scrib. **a**, Confocal section of cellularizing embryo stained for Scrib (green) and the basolateral membrane marker Neurotactin (red). Scrib localizes at the basal tip of the ingrowing cell membranes. Apicobasal (A–B) axis is shown; apical orientation is up in all confocal images. **b**, Stage-8 embryo stained as in **a**. During gastrulation, Scrib relocates to the apicolateral region of the ectodermal

epithelium. Note the much lower levels of Scrib in the non-epithelial mesoderm below the ectoderm. **c**, Stage-15 embryo stained for Scrib (green) and the basolateral protein FasIII (red). No Scrib is found in the apical cell membrane. **d**, Instead, Scrib (green) is in a small plaque that is basal to the adherens junction, revealed by Arm (red). It colocalizes with the septate junction marked by Cor (red) in **e**.

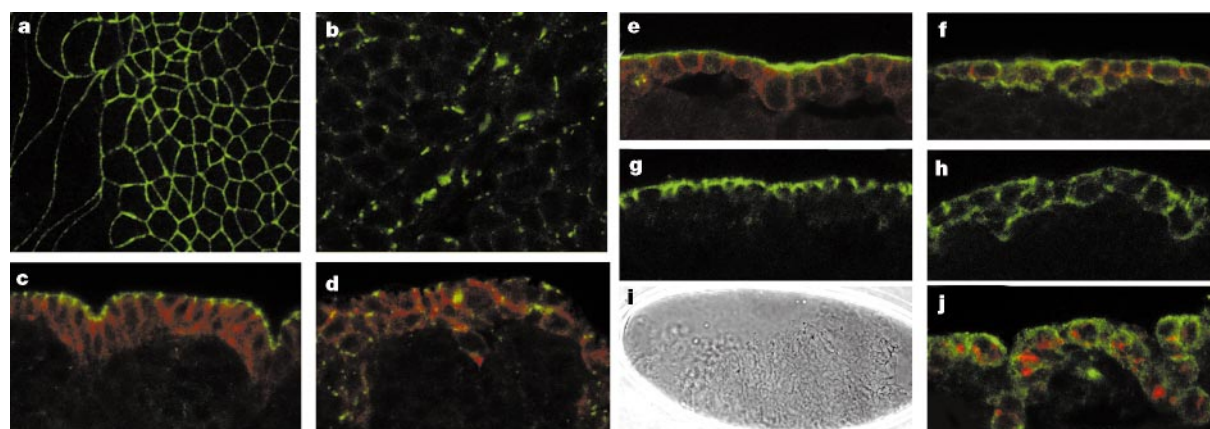


Figure 4 *scrib* embryos mislocalize apical proteins. Tangential confocal sections of wild-type (**a**) and *scrib* (**b**) stage-9 embryos stained for Arm. The regular apical band of Arm is severely disrupted in the mutant. Cross-sections of stage-13 wild-type (**c**) and *scrib* (**d**) epidermis stained for Arm (green) and Cor (red). In mutant tissues, Arm is found at ectopic sites around the surface of the cell instead of solely at the junction of contacting and non-contacting cell membranes. Note that Cor is not found at the apical surface of the *scrib* embryo. Cross-sections of wild-type (**e**) and *scrib* (**f**) stage-12 epidermis stained for Sas

(green) and Neurexin NrX (red). Sas is found basally and laterally as well as apically in the mutant, whereas NrX remains basolateral. Cross-sections of stage 10 wild-type (**g**) and *scrib* (**h**) epidermis stained for Crb. Again, Crb is found around basal and lateral cell surfaces as well as apical. **i**, Cuticle produced by embryo expressing ectopic Crb protein shows a similar morphology to *scrib* cuticles. **j**, Ectopic Crb-producing embryos show basolateral mislocalization of Dlt (green) but not apical mislocalization of Cor (red), similar to *scrib* embryos.

along the lateral cell membrane of ectodermal cells (Fig. 3b). The apicolateral epithelial staining seen at gastrulation continues into late embryogenesis, where it has consolidated into a narrow sub-apical region (Fig. 3c).

Because the subapical epithelial membrane is the site of cell–cell junctions, we determined whether Scrib localizes specifically to a junction. In mature *Drosophila* epithelia, the adherens junction and the septate junction (analogous to the vertebrate tight junction³) are adjacent structures that are located at the margins of the apical and basolateral surfaces respectively⁸. Co-staining for Scrib and the adherens junction marker Armadillo (Arm), the homologue of vertebrate β -catenin, reveals that Scrib is located immediately basal to the adherens junction (Fig. 3d), whereas co-staining for Scrib and the septate junction marker Coracle (Cor), a protein 4.1 family member, reveals coincident protein distributions (Fig. 3e). These results show that Scrib localizes to septate junctions. Although the septate junction does not appear ultrastructurally until late embryogenesis at stage 14 (ref. 8), Scrib is enriched subjacent to the adherens junction after gastrulation at stage 8 (Fig. 3b). By contrast, the initial expression of Cor at stage 12 is spread throughout the basolateral membrane (Fig. 4c), with apicolateral enrichment visible only from stage 14. These data indicate that Scrib is an early marker for the site of the future septate junction, at the apical boundary of the basolateral compartment.

The septate junction localization of Scrib suggested that cell junctions might be defective in *scrib* mutants. We investigated the presence of cellular junctions using antibody probes specific for either the adherens or the septate junction. The initial assembly of adherens junctions in *scrib* cellular blastoderms is normal, but at stage 8, anti-Arm staining reveals a severe disruption of the forming circumferential adherens junction belts. The normally continuous ‘honeycomb’ pattern (Fig. 4a) seen in tangential apical sections of the epidermis is severely fragmented (Fig. 4b). Tissue cross-sections show that, rather than being lost, Arm is misdistributed. Instead of its wild-type localization exclusively at the apical tip of contacting cell membranes, much Arm is found around the cell periphery on the inside of the epidermis, in the midst of opposing cell membranes (Fig. 4d). At these sites, Arm colocalizes with E-cadherin (Ecad), the transmembrane component of the adherens junction (data not shown), indicating that adherens junctions form at ectopic basolateral membrane positions in *scrib* embryos.

The formation of adherens junctions at ectopic positions within *scrib* cells raised the issue of whether these cells have lost polarity. In the wild-type epidermis, apical proteins such as Stranded at Second (Sas) are localized to the non-contacting cell membrane at the surface of the embryo, whereas basolateral proteins such as Fasciclin III (Fas III) are found in a complementary domain along the contacting membranes of cells (Fig. 4e; Table 1)¹. In *scrib* epidermal cells at stage 11, Sas is distributed throughout the plasma membrane, in both contacting and non-contacting cell surfaces (Fig. 4f). By contrast, Fas III localization is much less affected; in particular, it is not significantly mislocalized into apical regions. This preferential loss of apical restriction was seen with a panel of both cytoplasmic

and transmembrane proteins through mid-embryogenesis (Table 1), after which late embryos repolarize some apical markers by a *scrib*-independent pathway (data not shown). Thus, the polarity defects in *scrib* epithelial cells arise not from a total loss of cell polarity but from a specific misdistribution of apical proteins.

To place Scrib within the known pathway for *Drosophila* epithelial polarity determination, we examined the effect of *scrib* mutations on Crumbs (Crb). Crb is an apically localized transmembrane protein (Fig. 4g) that is necessary and sufficient to confer apical character on plasma membrane¹⁹. In *scrib* embryos, Crb shows unrestricted localization in both apical and basolateral regions (Fig. 4h). Discs-lost (Dlt), a second protein required for formation of the apical domain², is similarly mislocalized (Table 1). We considered whether *scrib* mutants are identical to a gain-of-function *crb* phenotype by comparing them with embryos in which GAL4-driven¹⁰ Crb is present throughout the cell membrane. Ectopic Crb that is produced in this manner is sufficient to phenocopy several aspects of *scrib* embryos, including mislocalization of apical proteins and the cuticle pattern (Fig. 4i, j)¹¹. These data indicate that a major function of Scrib in epithelial polarity is to exclude Crb from the basolateral domain. As ectopic Crb does not cause the epithelial morphology and multilayering defects seen in *scrib* embryos, Scrib may be required for the localization of additional epithelial determinants as well.

Analysis of the morphological and polarization phenotypes exhibited by *scrib* embryos shows that Scrib is a critical component of epithelial architecture in the *Drosophila* ectoderm, and suggests that its function is closely linked to that of Crb. Scrib, like Crb, is not required for the early localization of basal Dlt or apical Arm during blastoderm formation, and *scrib* mutants do not exhibit the defective cellularization or precipitous loss of cell adhesion seen when Dlt or Arm, respectively, are depleted in the embryo^{2,12}. The increasingly severe cell shape, polarity and epithelial organization defects of *scrib* embryos are first manifested after gastrulation, coincident with the onset of defects in *crb* and *sdt* embryos. Loss of Crb results in loss of apical proteins from the plasma membrane and a failure to consolidate early adherens junction material into an apical band of ZAS^{11,13}, while in *scrib* embryos early adherens junctions become misdistributed basolaterally. Together with the similarities between *scrib* loss-of-function and *crb* gain-of-function phenotypes, these data place Scrib and Crb in a pathway required for the progression from the initially differentiated blastoderm membrane domains into a fully polarized epithelium with mature junctions.

Our results show that the junctional protein Scrib specifically restricts apical membrane determinants to the apical cell surface. This restriction allows the proper segregation of apical and basolateral domain components, and the appropriate placement of the adherens junction, resulting in full epithelial polarization. How does Scrib, a putative scaffolding¹⁴ protein whose localization bounds the apical domain, dictate the proper confinement of apical proteins? Two models suggest themselves. Scrib could assemble a diffusion barrier that physically separates apical and basolateral compartments, similar to the ‘fence’ function proposed for the vertebrate tight junction¹⁵. To date, such a barrier has been shown to exist only for lipid diffusion in the outer leaflet of the plasma membrane^{16,17}. If *scrib* mutations disrupt such a mechanical barrier, then secondary retention systems must serve to maintain basolateral protein restriction from the apical cell surface¹⁸. An alternative is that Scrib has a role in the polarized targeting of transport vesicles carrying apical proteins. The junctional complex, and in particular the tight junction, has been proposed to be a key sorting site for a subset of Golgi-derived vesicles¹⁹. In this model, Scrib might interact with the ‘exocyst’, a secretory targeting apparatus localized to the tight junction and involved in polarized segregation of transmembrane proteins²⁰. PDZ domain proteins have been implicated at several different sites of the protein trafficking pathway^{21,22}, and occasional punctate intracellular staining of Scrib (data not shown)

Table 1 Localization of proteins in *scrib* embryos, stages 8–13

Protein	Topology	Localization in wild type	Localization in <i>scrib</i>
Crb	TM	A/J	A + BL
Ecad	TM	A/J	A + BL
Arm	Cyto	A	A + BL
Sas	TM	A	A + BL
Dlt	Cyto	A	A + BL
Nrt	TM	BL	BL
β Spec	Cyto	BL	BL
FasIII	TM	BL	BL (reduced)
Nrx	TM	BL	BL (reduced)
Cor	Cyto	BL	BL (reduced)

TM, transmembrane; Cyto, cytoplasmic; A, apical; J, junctional; BL, basolateral.

is reminiscent of vesicles. Distinction between these models will rely on the identification of binding partners for Scrib. □

Methods

Fly stocks and cloning

scrib was mapped through assays of the ability of Y; autosome translocation chromosomes to provide paternal rescue of the *scrib* cuticle phenotype. About 15% of gametes from *T(Y;3)B158* males did not rescue the *scrib* germline clone (GLC) phenotype, whereas all gametes from *T(2;3)8r9* males did. This analysis limited the *scrib* region to 97BC, and a deficiency within this region (*Df(3R)Tl-x*) failed to complement *scrib*.

Non-complementing mutations were obtained from several collections; these new *scrib* alleles included two P-element-induced alleles. Lethal phase analysis of the mutations and *Df(3R)Tl-x* indicates that the ethylmethane sulphonate-induced alleles *scrib*¹ (formerly *7c10*; D.B. and N.P., unpublished data) and *scrib*² (formerly *l(3)673*; K. Anderson, unpublished data) act as genetic nulls. *scrib j7B3* and *scrib s42405*^{23,24} are induced by *P(lacW)* insertion. *scrib*¹ and *scrib*² were recombined onto *FRT82B* chromosomes for GLC production. The A2 cDNA was isolated from an embryonic cDNA library²⁵, and cloned into the vector pCasper for transgenic rescue experiments. For ectopic production of Crb, the strains *daGAL4* and *UAS-crb*¹ were crossed at 29 °C.

Antibody production and embryonic analysis

Peptides corresponding to N-terminal (RYSRTLEELFLDANHIRDLPKNF) and C-terminal (VDAEDMRNPLDEIEAVEFRS) portions of Scrib were used to immunize rabbits (QCB, Hopkinton, MA). An affinity-purified serum gave similar staining through mid-embryogenesis to that seen with the unpurified sera used here; this staining is absent in *scrib* embryos. Western blots were carried out using standard techniques with 100 embryos from 0–2-h collections of *y w* or *scrib* GLCs. Antibodies were obtained as follows: anti- α -Spectrin from D. Kiehart; anti-Sas from D. Cavener; anti-Dlt, anti-Neurexin and anti-Crb from M. Bhat; anti-Cor from R. Fehon; anti-Ecad from H. Oda; anti-Neurotactin, anti-FasIII and anti-Arm from the Developmental Studies Hybridoma Bank. Secondary antibodies were from Jackson Labs. Fixation and staining was done as described²⁶, except that 5' fixation periods were used for anti-Scrib staining. Embryos were staged according to standard methods²⁷, and assayed for polarization prior to stage 14. Fluorescent images were collected on a Leica TCS confocal microscope. Scanning electron micrographs were performed as described²⁸.

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A tripeptide 'anticodon' deciphers stop codons in messenger RNA

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The two translational release factors of prokaryotes, RF1 and RF2, catalyse the termination of polypeptide synthesis at UAG/UAA and UGA/UAA stop codons, respectively^{1–3}. However, how these polypeptide release factors read both non-identical and identical stop codons is puzzling⁴. Here we describe the basis of this recognition. Swaps of each of the conserved domains between RF1 and RF2 in an RF1–RF2 hybrid led to the identification of a domain that could switch recognition specificity. A genetic selection among clones encoding random variants of this domain showed that the tripeptides Pro-Ala-Thr and Ser-Pro-Phe determine release-factor specificity *in vivo* in RF1 and RF2, respectively. An *in vitro* release study of tripeptide variants indicated that the first and third amino acids independently discriminate the second and third purine bases, respectively. Analysis with stop codons containing base analogues indicated that the C2 amino group of purine may be the primary target of discrimination of G from A. These findings show that the discriminator tripeptide of bacterial release factors is functionally equivalent to that of the anticodon of transfer RNA, irrespective of the difference between protein and RNA.

A tRNA-like property has been speculated for release factors^{5,6} in reading stop codons, and our aim was to identify a putative peptide anticodon equivalent in release factors. From sequence comparisons, we have previously proposed a seven-domain structure for release factors (domains A–G; Fig. 1a, top). We swapped these seven domains combinatorially between RF1 (UAG-specific) and RF2 (UGA-specific) to screen for active release-factor hybrids that would display altered codon specificity *in vivo*. For this purpose, common restriction sites were introduced into a clone at, or near, the sequences encoding the domain junctions so that these base changes would not affect activity (Fig. 1a, bottom). We examined a combinatorial set of 128 release-factor hybrids for their ability

to complement RF1 knockout (*prfA::Km^R*) and RF2 knockout (*prfB::Cm^R*) alleles (see Methods). For the initial screening, phenotypic assessment of RF1 and RF2 activity was more reliable than *in vitro* analysis because of the difference in their specific activities and the occurrence of peptide-anticodon-independent omnipotent variants that recognise all three stop codons⁷.

These release-factor hybrids were scored in pair-wise combinations in which only one domain differed. The criterion used to assign the discriminator domain was its ability to switch, without exception, the complementation activity exclusively and efficiently between RF1 and RF2. We found that a unique set of functional release-factor hybrids, designated Ψ RF1 and Ψ RF2, containing a single distinct domain, domain D (Fig. 1b), showed RF1- and RF2-specific *in vivo* complementation (see Fig. 2b) and *in vitro* peptide-release activities (see Fig. 3a, b). Any active release-factor hybrid other than these two switched the selectivity upon substituting domain D between RF1 and RF2 (data not shown). We concluded that domain D contains the 'peptide anticodon'. We also concluded that the hybrid-release-factor sequence, referred to as Ψ RF (see Fig. 1b, bottom), can play a vital role in the assessment of the discriminator activity upon swapping the peptide anticodon segment between RF1 and RF2. The native RF1 and RF2 sequences were simply inactivated by the same swap (data not shown). Switching the RNA-recognition specificity with a peptide swap in tRNA synthetases works using a hybrid⁸ or non-hybrid⁹ construct depending on the enzymes.

The discriminator domain was further defined by swapping peptides within domain D. Construction of a series of intradomain release-factor hybrids permitted assignment of discriminator activity

to the central 15-amino-acid segment located in one of the most conserved regions. Five out of six residues in this segment differ and are perfectly conserved in RF1s and RF2s of many species (Fig. 2a; see ref. 6). Transfer of a 24-residue segment containing these 15 amino acids from RF1 to Ψ RF2 switched the specificity from RF2 to RF1 (Ψ RF1 β ; Fig. 2a; see Fig. 3c). Subsequent experiments used variants of this 15-residue segment as discriminator cassettes in hybrid domain D (Fig. 1b, bottom). The conserved RF1- and RF2-specific motifs in this region were 'Q-vPaTE- - -I' and 'V-kSPFD- - -R', respectively, where lower case represents less conserved, and upper case represents highly conserved, residues (Fig. 2a). Primary screening of the requirements for the discriminator by site-directed and combinatorial mutagenesis indicated that substitutions of amino acids other than 'PaT' and 'SPF' between these two motifs did not affect the selectivity of codons (data not shown). Hence, we used 'Q-VXXXX- - -I' as a universal cassette motif, for which tripeptide XXX was extensively analysed.

The systematic selection of the discriminator motifs was carried out using designed tripeptide collections in which the first or third position was fixed as an amino acid of RF1 and RF2 in otherwise (partly) random sequences (see Fig. 2a, bottom): PX₄X₂₀ (pool-1), SX₄X₂₀ (pool-2), X₂₀X₄T (pool-3), X₂₀X₄F (pool-4), and SX₂₀T (pool-5), where P = Pro, S = Ser, T = Thr, F = Phe, X₄ = Ala/Pro/Ser/Thr, and X₂₀ = 20 amino acids (Table 1). Winners from pool-1 in the RF1-null strain contained any of four residues at the second position but mostly threonine (56 out of 78). Less frequently, other non-charged amino acids were at the third position, and no winners appeared in the RF2-null strain. Winners from pool-2 in RF1 and RF2-null strains contained proline at the second position (note that alanine and serine were also allowed in RF1) and mostly shared

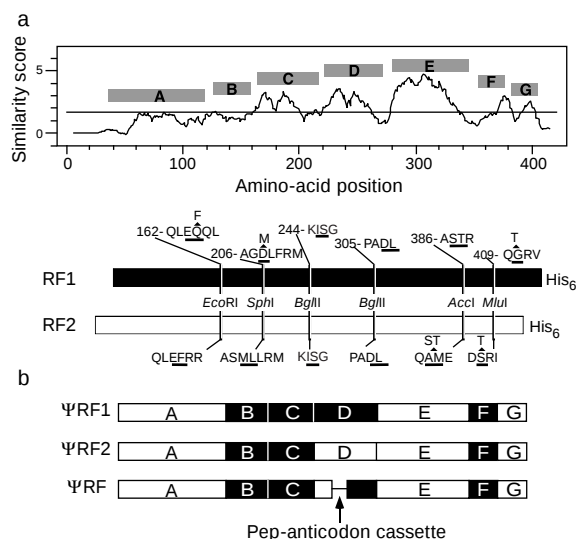


Figure 1 A seven-domain model of release factors and release-factor hybrids developed for the pep-anticodon assignment. **a**, The average similarity plots of 22 bacterial RF1 and RF2 sequences and the proposed seven conserved domains A–G (ref. 6). Restriction-enzyme sites were generated at or near the domain junctions whose relevant peptide sequences are shown. Amino acids for which restriction sites were designed are underlined: most of the nucleotide-sequence changes did not alter amino acids except in five positions (changes are marked by arrows) that altered single (non-conserved) residues. The amino-acid position refers to the coordinate of the similarity alignment (ref. 6), which is distinct from those of native RF1 and RF2. **b**, A pair of release-factor hybrids, Ψ RF1 and Ψ RF2, that exerted RF1- and RF2-specific complementation activities owing to the respective domain D inserts. A hybrid release-factor construct, designated Ψ RF, was used as a test release-factor backbone to monitor the specificity of transplanted peptides and an amino-acid swap made in the discriminator tripeptide. Closed and open boxes represent RF1 and RF2 segments, respectively.

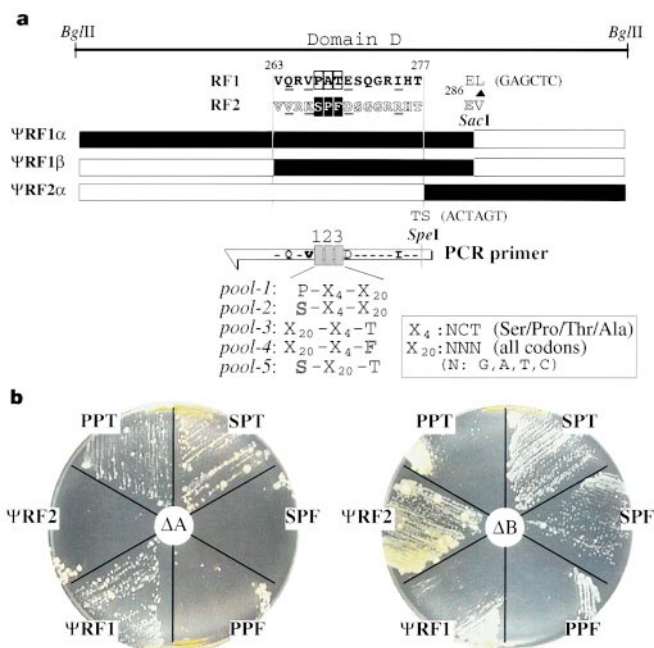
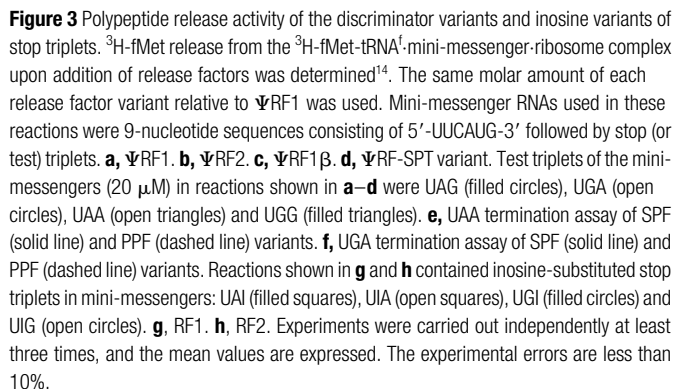


Figure 2 The assessment of the discriminator tripeptide within domain D. **a**, Intradomain D hybrids that exerted RF1- and RF2-specific complementation activities owing to the respective mosaic intervals. These hybrid sequences were transplanted into the Ψ RF construct and examined for their specificity by *in vivo* complementation and *in vitro* release assay. The amino-acid sequence of the assigned discriminator locus is shown. The discriminator tripeptides are boxed and other conserved residues are underlined. Restriction sites for peptide transplantation are shown with the relevant dipeptide (arrow indicates that the amino acid is changed). PCR primers designed for random pools of the discriminator tripeptides are indicated (see Table 1). **b**, Growth of RF1-null (Δ A, RM789A; left panel) and RF2-null (Δ B, RM789B; right panel) strains transformed with pBAX-RF derivatives carrying: Ψ RF1, Ψ RF2, PPT, SPT, SPF and PPF tripeptide variations.



amino acids at the third position (these tripeptides acquired omnipotence for three stop codons as shown below) except for phenylalanine which was restricted to the RF2-null strain. Winners from pool-3 in the RF1-null strain contained serine or proline at the first position and any four residues at the second position, and the only winner in the RF2-null strain was Ser-Pro-Thr (omnipotent, see below). When the third position is fixed as phenylalanine (pool-4), the sole winner was Ser-Pro-Phe which appeared in the RF2-null strain. Growth of the RF1- or RF2-null strains in the presence of representative tripeptide variants, PPT (RF1-type), SPT (omnipotent), SPF (RF2-type) and PPF (see below), is shown in Fig. 2b. These and other *in vivo* complementation data are summarized in Table 1.

a

Pep-anticodon tripeptide

1st position 3rd position

Ser Thr

G G

A A

A A

Pro Phe

2nd base 3rd base

discriminator discriminator

RF1

RF2

Omnipotent

UAA restricted

b

NC1=NC=NC2=C1N=CN2

O=C1NC=CC2=C1N=CN2

NC1=NC=NC2=C1N=CN2

NC1=NC=NC2=C1N=CN2

tripeptide in RFI is less restrictive, and that proline is effective, hence further manipulations of the tripeptide included proline at the second position (as shown in Fig. 3e, f). The double-switch model for stop codon discrimination is validated and clarified further by two lines of evidence. First, the substitution of Pro-Pro-Phe for Ser-Pro-Phe in an otherwise ΨRF2 backbone reduced UGA termination but had virtually no effect on UAA termination, even with a large amount of this variant (compare Fig. 3e, f). Second, the omnipotent tripeptide Ser-Pro-Thr also conferred peptide release activity with UGG as potential stop codon (Fig. 3d). These results led us to conclude that the defined tripeptide represents the discriminator element of the peptide anticodon; hereafter referred to as ‘pep-anticodon’. We believe that this is a naturally functioning pep-anticodon, not a hybrid-specific one, because of the hybrid context and because the tripeptide is made up entirely of the sequences of the natural release factors.

What atoms or atomic groupings of purine bases are discriminated by the discriminator tripeptide? In principle, two side groups may be potent targets, either C2 amino (G) discriminated from C2 proton (A) or C6 carbonyl (G) discriminated from C6 amino (A), or both (Fig. 4b). To test these possibilities, we substituted inosine (I; see Fig. 4b) for G in the stop codon and analysed polypeptide

Table 1 Selection of discriminator tripeptides of pep-anticodon

Selected pool	Winners in RF1-null strains (score/specificity)		Winners in RF2 null-strains (score/specificity)	
Pool-1	(78 clones scored)		(no clones selected)	
PX ₄ X ₂₀	PTT (20/A) PST (12/A) PPN (8/A) PSN (3/A) PTS (2/A) PSQ (1/A)	PAT (16/A) PPT (8/A) PTN (3/A) PPS (3/A) PPL (2/A)	(none)	
Pool-2	(50 clones scored)		(49 clones scored)	
SX ₄ X ₂₀	SPT (19/AB) SPL (7/AB) SPI (1/AB) SST (1/AB)	SPR (19/AB) SAT (2/AB) SPH (1/AB)	SPT (19/AB) SPI (7/AB) SPL (2/AB) SPH (1/AB)	SPR (16/AB) SPF (3/B) SPM (1/AB)
Pool-3	(68 clones scored)		(68 clones scored)	
X ₂₀ X ₄ T	SPT (18/AB) SAT (13/A) PTT (8/A) STT (2/A)	PST (14/A) PPT (9/A) PAT (4/A)	SPT (68/AB)	
Pool-4	(no clones selected)		(34 clones scored)	
X ₂₀ X ₄ F	(none)		SPF (34/B)	
Pool-5	(81 clones scored)		(41 clones scored)	
SX ₂₀ T	SPT (19/AB) SRT (8/A) STT (6/A) SST (5/A) SVT (3/A) SGT (3/A) SNT (2/A) SDT (1/A)	SAT (9/A) SLT (7/A) SHT (6/A) SIT (4/A) SKT (3/A) SQT (2/A) SFT (2/A) SMT (1/A)	SPT (41/AB)	

ΨRF variants carrying designed (random) sequence pools of discriminator tripeptides were transformed into RF1-null and RF2-null strains, and active variants were selected (see Methods). These winners were examined for their sequence and activity to complement RF1- and/or RF2-null mutations. The number of winners scored and the specificity are shown in parentheses. Specificity: A, RF1 specificity (*prfA* complementation); B, RF2 specificity (*prfB* complementation); AB, omnipotent specificity (*prfAB* double mutant complementation). Amino acids are in single-letter code. X₄ represents Ala/Pro/Ser/Thr; and X₂₀ represents 20 amino acids.

release *in vitro*. Both UAI and UIA were recognized by RF1 and RF2 (Fig. 3g, h), showing that the inosine substitution removed the primary discrimination target. On the other hand, UIG and UGI were still recognized selectively by RF1 and RF2, respectively (Fig. 3g, h). Therefore, we believe that the pep-anticodon discriminates primarily the C2 amino group of G at both second and third bases. Nevertheless, the potential contribution, if any, of the C6 amino group of A remains to be examined because of the apparently lower release activity of A-to-I variants at the discriminator position (see Fig. 3g, h).

The principal rule of base discrimination by the discriminator tripeptide is that amino acids used for restricted recognition of A are bulky hydrophobic residues, such as proline and phenylalanine, whereas those for relaxed recognition of A and G are small hydrophilic residues, such as serine and threonine. The latter, relaxed recognition of A and G may be phenotypically equivalent to wobble pairing between the messenger RNA triplet and tRNA anticodon at the third position^{10,11}. What is unique to release factors is allowing wobble not only at the third position of the stop codon but also at the second position. It is of interest that in eukaryotes a single release factor, eRF1, recognizes all three stop codons. An omnipotent discriminator tripeptide, such as Ser-Pro-Thr, cannot account for the three-codon-specific recognition because it recognizes UGG as well. Therefore, eRF1 must have its own discrimination mechanism: the simplest way to achieve this would be to permit two purine bases to exist at the second and third positions, except for double G, in a single recognition site. Our identification of the discriminator tripeptide strongly suggests that release factors recognize stop codons directly rather than indirectly through the interplay of stop codons and rRNA sequences¹². The amino acid(s) responsible for recognition of the first base, U, of the stop codon must be the adjacent residue(s) conserved in both RF1 and RF2. In summary, our discovery of the discriminator tripeptide, or pep-anticodon, in bacterial release factors clearly solves the long-standing coding

problem of how a release factor reads a stop codon. Moreover, it is important to point out that our experimentally defined discriminator tripeptide agrees with the predictions of our release-factor tRNA mimicry hypothesis^{2,6}. □

Methods

Plasmids and manipulations

Plasmid pBAX-RF is a pBR322-derived RF expression plasmid⁷ in which release-factor hybrids or those with domain cassettes are inserted into *Bam*HI–*Sac*I (replaced for *Nru*I) sites and expressed constitutively from the *tet* promoter. The release-factor hybrids contained a carboxy-terminal histidine tag encoded by a sequence generated by PCR primers as described¹³. Intervals of domains A–G of RF1 and RF2 were marked with restriction enzyme sites using designed PCR primers (see Fig. 1a). These artificial restriction sequences did not affect release-factor activity. Interdomain swapping was carried out by amplification of the appropriate DNAs from RF1 and RF2, followed by substitution of their restriction fragments. Intradomain swapping was achieved using PCR primers that were designed to encode hybrid release-factor sequences.

In vivo complementation and selection

Escherichia coli W3110 derivatives^{7,14}, RM789A (*prfA*::Km^R), RM789B (*prfB*::Cm^R) and RM789AB (*prfA*::Km^R *prfB*::Cm^R), carrying plasmid pSUIQT-RF2*⁷, were used as indicator hosts. pSUIQT-RF2* is a *lac* promoter-controlled expression plasmid of an RF2 variant, RF2*, that is able to recognize UAA/UGA/UAG; hence, it complements the single or double RF1–RF2 knockout alleles in the presence of isopropyl-1-thio-β-D-galactoside (IPTG). For complementation tests, indicator strains were transformed with two compatible plasmids, pSUIQT-RF2* and a test pBAX-RF construct, in the presence of IPTG, and growth was monitored in the absence of IPTG. For selections, pSUIQT-RF2*-bearing indicator cells were transformed with pBAX-RF hybrid or cassette pools, and survivors were selected in the absence of IPTG to give rise to pBAX-RF winners. These results were consistent with those derived from temperature-sensitive alleles in RF1 (*prfA1*)¹⁵ and RF2 (*prfB286*)¹⁶ (data not shown).

Peptide pools

Randomized collections of the discriminator tripeptide encoded in 76-mer primers (antisense; 5'-GGGGATCCACTAGTATGAATGCGACCTGGGAGTCNNNCGNNN-NAACACGTTGCACACGATGTACGCCGGTTTCAG-3' where N indicates A/G/T/C mix) carrying designed sequence variations in domain D were used to amplify ΨRF segments containing domains A–D by PCR using the 5' flanking vector sequence (5'-CGAC-

TACGCGATCATGGCGAC-3'). The amplified DNA pools were digested with *Bam*HI and *Spe*I restriction enzymes, and inserted into the tester plasmid pBAX-ΨRF (see Fig. 2a, bottom).

Protein purification and peptide release assay

Histidine-tagged release-factor genes were cloned downstream of a T7 RNA polymerase promoter in plasmid pET30a (Novagen, Inc.), and overexpressed in *E. coli* BL21 (DE3) cells by addition of 0.5 mM IPTG for 2.5 h as described^{7,13,14}. Histidine-tagged release-factor proteins were purified to homogeneity from cell lysates by affinity chromatography using Ni-NTA Agarose (Qiagen). The ability of purified release-factor hybrids to terminate protein synthesis was monitored by the rate of *N*-formylmethionine (fMet) release at the stop codon from the *in vitro* termination complex composed of ³H-fMet-tRNA^f, 9-nucleotide mini-messenger (5'-UUCAUGNNN-3' where NNN is a test triplet) and the ribosome isolated from *E. coli* essentially as described^{7,14}.

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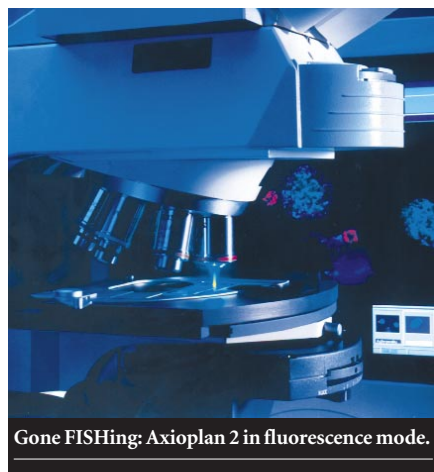
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Real-time image recording and analysis

FLIR Systems are promoting the combination of their new cordless ThermaCAM SC2000 thermographic camera and Tracer Plus PC-based real-time image recording and analysis package, for thermal analysis in



Get real: FLIR System's imaging package.



Gone FISHing: Axioplan 2 in fluorescence mode.

scientific IR applications. The palm-sized ThermoCAM SC2000 features a solid-state microbolometer sensor which needs no cryogenic cooling. When linked to Tracer Plus, the ThermoCAM SC2000 feeds real-time data directly to the PC for immediate analysis.

Reader Service No. 106

Axioplan 2

Zeiss

www.zeiss.de

New ideas for multichannel fluorescence microscopy

The Axioplan 2 microscope is optimized for fluorescence applications, including FISH (fluorescence *in situ* hybridization) and M-FISH techniques in genetics and for multichannel fluorescence applications. A new fluorescence system allows eight different fluorescence images to be recorded manually or automatically controlled by computer. Novel features include an 8-position filter turret with an unrestricted field of 25 mm to

save time during sample screening.
Reader Service No. 107

COMPex lasers

Lambda Physik

www.lambdaphysik.com

New lasers, available to order

Multigas versions are now available for COMPex and LPX 200 lasers. A multigas excimer laser provides the flexibility of selecting any of the four wavelengths (193, 248, 308 and 351 nm) while using the same laser tube. So one optimized multigas system replaces optimized versions for chlorine and fluorine. Lambda Physik's NovaTube metal/ceramic design virtually eliminates component corrosion and gas contamination while increasing gas life and extending maintenance intervals.

Reader Service No. 108

PicoStar HR

LaVision

www.lavision.com

'State of the art' CCD cameras

This ultrahigh repetition rate, fast-gated intensified CCD camera is recommended for use in conjunction with high repetition rate mode-locked lasers. Typical applications include: time-resolved imaging and spectroscopy, fluorescence lifetime imaging microscopy, single-molecule imaging, multifocal multiphoton microscopy, quantum dot imaging, fluorescence resonance energy transfer microscopy, time-gated imaging and optical tomography. The camera's features include: optical gate width down to 200 ps, gating repetition rate up to 110 MHz, gain modulation up to 1 GHz; UV-NIR spectral range, single-photon sensitivity, 2-D photon counting mode, dynamic range up to 16 bit, and high spatial resolution. The system is computer controlled and comes with Windows-compatible software for peripheral-device control (including laser, spectrograph, filter, shutter, camera functions), image acquisition, processing and data-analysis routines. An upgrade allowing exposure time down to 50 ps is available.

Reader Service No. 109

Molecular biology poster

Eppendorf

www.eppendorfscl.com

A colourful way of covering 70 × 100 cm of wall space

This poster, "Molecular Biology", is produced by Eppendorf-Netherler in collaboration with Springer, Heidelberg. It shows the components of a eukaryotic cell as well as graphic illustrations of the structure of DNA, transcription, DNA replication, translation, protein construction, mutations and the principles of mutation analysis. The full-colour poster is printed in English and is available free of charge from Eppendorf.

Reader Service No. 110



SPOT colour: SPOT RT *in situ*.

SPOT RT Colour

Diagnostic Instruments

www.diagine.com

Digital cameras designed for use in microscopy

The SPOT RT Color (Real Time Camera) is equipped with dual circuitry for real-time monochrome viewing with 19 f.p.s. at 760 × 540 resolution or high-accuracy imaging with 12-bit, 6-MHz readout at 1,520 × 1,080 resolution. Cooling to -12 °C ensures low noise operation. Threshot RGB colour technology provides 36-bit non-interpolated colour images. The filter also has a clear mode that provides 12-bit non-interpolated monochrome images. The SPOT software includes facilities for advanced editing, sequential imaging, calibration-mark insertion, feature measurement, image archiving and report generation.

Reader Service No. 111

Blue Microlaser

Melles Griot

www.mellesgriot.com

A compact, high-power, solid-state laser system

The new Blue Microlaser delivers up to 400 mW of single-frequency power at 457 nm, with low-amplitude noise and good beam-pointing stability. Power input is approximately 60 W of electrical power at room temperature. Applications include bio-medical diagnostics, Raman and fluorescence spectroscopy, confocal and fluorescence microscopy, and laser display.

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These notes are compiled in the Nature office from information provided by the manufacturers. For more details, fill in the reader service card.

Check out our homepage at <http://www.resgen.com>

Peptides
\$14/residue
10 residue minimum

Polyclonal Antibodies - \$1,195

Please contact us about complete services for your custom peptide and polyclonal antibody needs.

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U.K. 0-800-89-1393
FAX 256-536-9016

READER ENQUIRY NO. 4